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BONE MERROW MESENCHYMAL STEM AND PROGENITOR CELLS IN MYELOID MALIGNANCIES

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THESIS FOR DOCTORAL DEGREE (Ph.D.)

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“I wonder if we might pledge ourselves to remember what life is really all about—not to be afraid that we’re less flashy than the next, not to worry that our influence is not that of a tornado, but rather that of a grain of sand in an oyster! Do we have that kind of patience?”

— Fred Rogers

To my dear parents
献给我亲爱的父母

ABSTRACT

Hematopoietic stem cells (HSCs) reside in bone marrow (BM) microenvironment / niche to self-renew and generate all types of mature blood cells in postnatal life. This process is called hematopoiesis. Normal hematopoiesis is tightly regulated by both intrinsic signaling in HSCs and extrinsic signals from BM niche. Despite the involvement of BM niche in normal and malignant hematopoiesis has been reported, how different BM cellular niche contribute to leukemia progression and how leukemia cells affect their BM niche remain largely unknown.

The overall aim of this thesis was to determine the roles of BM niche cells in the regulation of different myeloid malignancies, and understand the underlying mechanisms. In **study I**, by taking advantage of *Sipa1*$^{-/-}$ mouse model, we revealed that the loss of Sipa1 in mice led to BM niche alterations before the onset of MDS/MPN, and confirmed that the loss of Sipa1 in BM niche, but not in hematopoietic cells led to the MDS/MPN. Moreover, we detected abnormal inflammatory cytokines and growth factors in the BM stromal cells of the disease-free young adult *Sipa1*$^{-/-}$ mice, which could contribute to the pathogenesis of the MDS/MPN in aged *Sipa1*$^{-/-}$ mice. This study provides evidence for BM niche-induced MDS/MPN and the underlying molecular and cellular mechanisms.

Given the distinct pathogenesis of different types of myeloid malignancies, we further investigated the contributions of BM niche to AML progression using MLL-AF9^{+} AML mouse model in **study II**. We detected alterations of BM niche cells in symptomatic AML mice, and confirmed similar alterations of BM stromal cells in the NSG mice engrafted with primary patient AML cells. Furthermore, BM niche cells in the AML mice produced altered growth factors, cytokines, and matrix proteins. Dynamic BM niche cell analysis and gene expression assay revealed that the BM niche alterations correlated with AML burden. We next functionally determined the roles of BM MSCs marked by Ebf2 expression in AML progression. We demonstrated that the Ebf2^{+} cells participated in AML BM niche formation. Notably, the depletion of Ebf2^{+} cells accelerated AML development in mice, indicating the suppressive roles of native BM niche to AML development. This study indicated that AML cells educated normal BM niche into a reinforcing AML BM niche contributing to AML progression.

In **study III**, we found that BM MSCs altered their phenotype with reduced SCA1 expression in non-adherent culture, emphasizing culture-related alterations of BM MSC immunophenotype. In **study IV**, by using LTC-IC assay and single cell PCR, we revealed diverse expressions of leukotriene signaling molecules and minor effects of ALOX5 and CYSLT1 antagonists on CML LSC growth, particularly in the presence of BM stromal cells *in vitro*.

All together, we phenotypically, molecularly, and functionally characterized leukemic BM niche and determined the niche contributions to different myeloid malignancies. Targeting on BM niche factors or restore BM niche components might be new therapeutic strategies for these myeloid malignancies.

LIST OF SCIENTIFIC PAPERS

I. ***Sipa1*** **Loss-induced Bone Marrow Niche Alterations Drive Myeloproliferative Neoplasia.**
Pingnan Xiao, Monika Dolinska, Lakshmi Sandhow, Makoto Kondo, Anne-Sofie Johansson, Thibault Bouderlique, Ying Zhao, Xidan Li, Marios Dimitriou, Eva Hellström Lindberg, Nagahiro Minato, Julian Walfridsson, David T. Scadden, Mikael Sigvardsson, Hong Qian. *Manuscript*

II. **Distinct Roles of Mesenchymal Stem and Progenitor Cells During the Development of Acute Myeloid Leukemia in Mice**
Pingnan Xiao, Yaser Heshmati, Makoto Kondo, Thibault Bouderlique, Monika Dolinska, Lakshmi Sandhow, Anne-Sofie Johansson, Mikael Sigvardsson, Marja Ekblom, Julian Walfridsson, Hong Qian. *Manuscript*

III. **Nonadherent Culture Method Downregulates Stem Cell Antigen-1 Expression in Mouse Bone Marrow Mesenchymal Stem Cells**
Baoping Deng, Weiping Deng, Pingnan Xiao, Kuan Zeng, Shining Zhang, Hongwu Zhang, David Yb Deng and Yanqi Yang. *Experimental and therapeutic medicine*, 2015 Jul;10(1):31-36.

IV. **Leukotriene Signaling via *ALOX5* and Cysteinyl Leukotriene Receptor 1 Is Dispensable for in Vitro growth of $CD34^{+}CD38^{-}$ Stem and Progenitor Cells in Chronic Myeloid Leukemia.**
Monika Dolinska, Alexandre Piccini, Wan Man Wong, Eleni Gelali, Anne-Sofie Johansson, Johannis Klang, Pingnan Xiao, Elham Yektaei-Karin, Ulla Olsson Strömberg, Satu Mustjoki, Leif Stenke, Marja Ekblom, Hong Qian. *Biochem Biophys Res Commun*, 2017 Aug;490(2):378-384.

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LIST OF ABBREVIATIONS

5-LO	5-lipoxygenase
AML	Acute myeloid leukemia
ANGPTL1	Angiopoietin like 1
bFGF	Basic fibroblast growth factor
BM	Bone marrow
BMP	Bone morphogenetic protein
CAR cells	CXCL12-abundant reticular cells
CCL3	Chemokine ligand 3
CFU-F	Colony-forming unit fibroblast
CML	Chronic myeloid leukemia
CMP	Common myeloid progenitor
CNL	Chronic neutrophilic leukemia
Col	Collagen
Col1a1	Collagen type I alpha 1
CXCL12	CXC chemokine ligand 12
CXCL4	CXC chemokine ligand 4
CXCR4	CXC chemokine receptor type 4
DTR	Diphtheria toxin
Ebf2	Early B cell factor 2
ET	Essential thrombocythemia
FABP4	Fatty acid binding protein 4
FGF1	Fibroblast growth factor1
Flk1	Fetal liver kinase 1
Fmod	Fibromodulin
GFP	Green fluorescent protein
GMP	Granulocyte-macrophage progenitor
Grem1	Gremlin 1
HPC	Hematopoietic progenitor cell
HSC	Hematopoietic stem cell
IGF-1	Insulin-like growth factor 1
Igf1	Insulin-like growth factor 1
IL	Interleukin
JAK2	Janus kinase 2
JMML	Juvenile myelomonocytic leukemia
KITL	Kit ligand
LepR	Leptin receptor
LMPP	Lymphoid-primed multi-potential progenitor
LSC	Leukemic stem cell
LSK	$Lin^{-}SCA1^{+}cKIT^{+}$
LT-HSC	Long-term hematopoietic stem cell
LTC-IC	Long-term culture-initiating cell
mTOR	Mammalian target of rapamycin
MAPK	Mitogen-activated protein kinases
MCAM	Melanoma cell adhesion molecule
MDS	Myelodysplastic syndrome
MEP	Megakaryocyte-erythrocyte progenitor

Micro/μCT	Micro-computed tomography
MPN	Myeloproliferative neoplasms
MSC	Mesenchymal stem cell
Mx1	Myxovirus resistance-1
NG2	Nerual/glial antigen 2
NK	Nature killer
Nov	Nephroblastoma overexpressed
NSG	NOD scid gamma
Ocn	Osteocalcin
Opn/Spp1	Osteopontin
Osx/Sp7	Osterix
PDGF-BB	Platelet-derived growth factor-BB
PDGFRA/B	Platelet-derived growth factor receptor alpha/beta
PECAM-1	Platelet/Endothelial Cell Adhesion Molecule 1
PF-4	Platelet factor-4
PI3K	Phosphoinositide 3 kinase
PMF	Primary myelofibrosis
PPARγ	Peroxisome proliferator-activated receptor gamma
Prx1	Paired related homeobox gene 1
PTH	Parathyroid hormone
PV	Polycythemia vera
RANKL	Receptor activator of NFκB ligand
Rap1GAP	Rap1 GTPase-activating protein
RARγ	Retinoic acid receptor γ
ROS	Reactive oxygen species
Runx2	Runt-related transcription factor 2
Sbds	Shwachman-Diamond-Bodian syndrome
SCA1	Stem cell antigen 1
SCF	Stem cell factor
SIPA1	Signal-induced proliferation-associated gene-1
ST-HSC	Short-term hematopoietic stem cell
TGF-α/β	Transforming growth factor alpha/beta
TGFβ1	Transforming growth factor β 1
THPO	Thrombopoietin
THY1	Thymocyte differentiation antigen 1
TKI	Tyrosine kinase inhibitors
Vcam1	Vascular cell adhesion protein 1
VEGF	Vascular endothelial growth factor
VLA-4	Very late antigen-4

1 Introduction

1.1 Hematopoiesis and HSC niche

Normal hematopoiesis takes place in a series of various hematopoietic organs from the embryo stage to adult. Aorta-gonad-mesonephros, yolk sac, placenta, fetal liver, and spleen are involved in hematopoiesis during prenatal development, whereas bone marrow (BM) is the main organ for hematopoiesis after birth (Mikkola and Orkin, 2006). The concept of the BM hematopoietic stem cell (HSC) niche, a specialized BM microenvironment, was first proposed by Schofield with a view that the BM niche is critical for normal hematopoiesis (Schofield, 1978). The BM niche cells could secrete cytokines, growth factors, and matrix proteins, as well as mediate cell-cell interactions to regulate HSCs quiescence, proliferation, self-renewal, and differentiations.

1.1.1 Normal hematopoietic cell hierarchy

Under steady state, hematopoietic lineage cells, such as erythrocytes (red blood cells), platelets, granulocytes, monocytes, and lymphocytes, are differentiated from HSCs in BM and released into the blood circulation after maturation. These blood cells play their specific roles in oxygen delivery, stopping bleeding, and defense against pathogens, maintaining a homeostasis of our bodies.

Erythrocytes are hemoglobin-rich nucleus-free cells, working as the principal oxygen carriers to deliver oxygen throughout the body. Erythrocytes express Glycophorins A (CD235) in human (Merryweather-Clarke et al., 2011), and TER119 in mice (Koulnis et al., 2011). They develop from reticulocytes and are phagocytized by macrophages after a life span of around 120 days. Similar to erythrocytes, platelets are also nucleus-free blood cells. Platelets are tiny cytoplasmic fragments generated from megakaryocytes, and play a critical role in hemostasis via adhering and aggregating to damaged vessel walls, followed by the activation of coagulation cascades.

Granulocytes, including neutrophils, basophils, and eosinophils, are specific white blood cells with granules in their cytoplasm. They belong to the cells involved in the innate immune system, and defense against pathogen-mediated infections and inflammations. Mature granulocytes usually have multi-lobed nuclei and express CD11B and Gr1 on their cell surface. Monocytes are bigger than granulocytes and also express the cell surface marker CD11B but not Gr1. They can differentiate into macrophages, and defense against pathogen invasions together with granulocytes. Both granulocytes and monocytes are originally differentiated from BM HSCs, followed by common myeloid progenitors (CMPs) and granulocyte-macrophage progenitors (GMPs).

Lymphocytes are composed of natural killer (NK) cells, B cells, and T cells with a relatively long lifespan (possibly up to several years in human) compared with other mature white blood cells. NK cells of C57BL/6 or NZB strain can be recognized with the cell surface marker NK1.1 (Stenstrom et al., 2005), whereas human NK cells express CD56. NK cells

join in innate immune response and induce apoptosis of infected cells. Mature B cells express CD19 as their cell surface marker. T cells express CD3 and can be subdivided into $CD4^+$ helper T cells, and $CD8^+$ cytotoxic T cells based on the different antigen expression on their cell surface after positive and negative selections in thymus. B cells and T cells participate in adaptive immunity to defend against antigens and pathogens through antibody- or cell-mediated immune reactions.

HSCs, the precursors of all mature blood cells located at the top of hematopoietic hierarchy, replenish downstream lineage cells and maintain hematopoietic homeostasis (**Figure 1**). In mouse, HSPCs express CD45, CD117 (c-KIT), and stem cell antigen 1 (SCA1) (Adolfsson et al., 2001) and are devoid of lineage markers (Lin), including TER119, CD11B, Gr1, CD3, CD4, CD8, CD19, and NK1.1. Therefore, BM HSPCs reside in $Lin^-SCA1^+c\text{-}KIT^+$ cells (LSKs). HSPCs can be subdivided into long-term HSCs (LT-HSCs) with a phenotype of $LSKFlt3^-CD34^-CD150^+$, short-term HSCs (ST-HSCs) as $LSKFlt3^-CD34^+$, and lymphoid-primed multi-potential progenitors (LMPP) as $LSKFlt3^{high}CD34^+$ (Adolfsson et al., 2001; Christensen and Weissman, 2001; Kiel et al., 2005; Yang et al., 2005). Hematopoietic progenitor cells (HPCs) lack the SCA1 expression (LSK^-). With the combinations of the markers of CD16/32 and CD34, HPCs are further divided into GMPs, CMPs, and megakaryocyte-erythrocyte progenitors (MEPs), presenting the phenotypes of $LSK^-CD16/32^+CD34^+$, $LSK^-CD16/32^-CD34^+$, and $LSK^-CD16/32^-CD34^-$, respectively. MEP may also be differentiated directly from HSCs (Pronk et al., 2007). CMPs give rise to GMPs and MEPs, and generate down-streams of granulocyte/monocyte and megakaryocyte/erythrocyte lineages (Akashi et al., 2000)

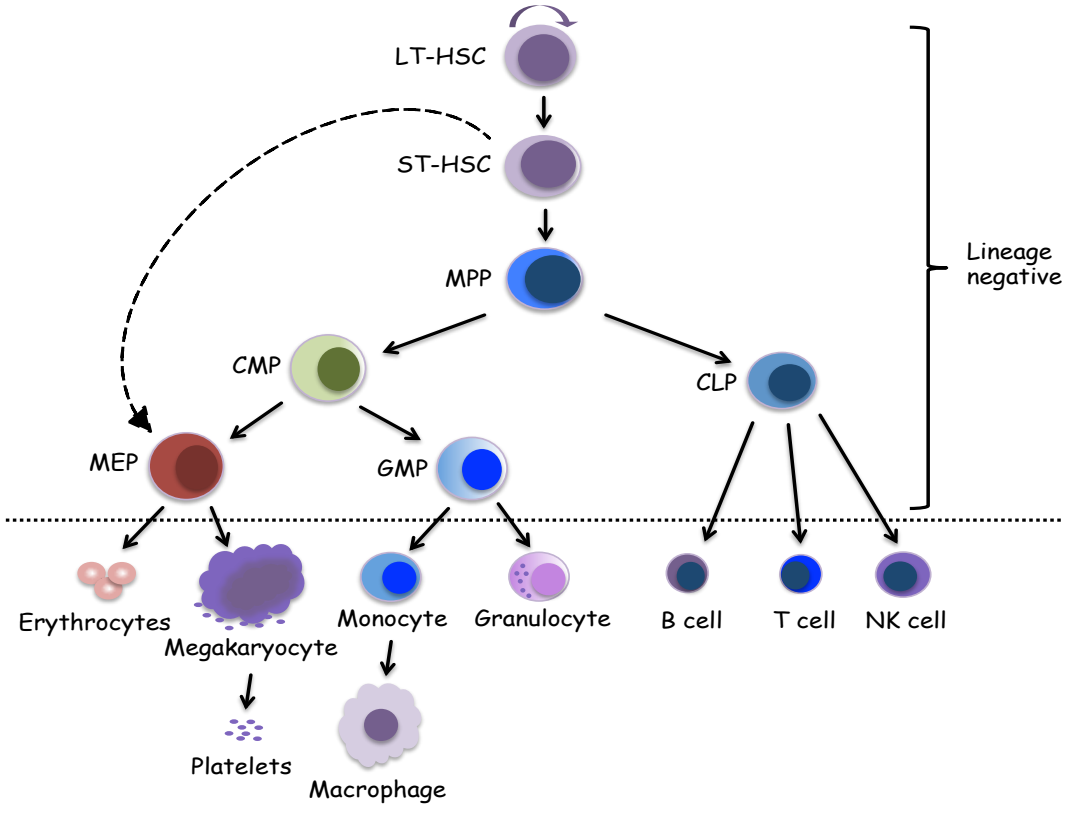

Figure 1. Developmental hierarchy of murine hematopoietic cells. LT-HSCs give rise to hematopoietic progenitors and mature hematopoietic cells as described above.

1.1.2 Regulations of hematopoiesis by BM microenvironment

HSCs are located in the specific BM niche during postnatal life to self-renew and produce progenies. The anatomic architectures of the bone from the outer layer to the inside of this special cavity are periosteum (a membrane of connective tissue), cortical bone (hard bone), trabecular bone (spongy bone), and central marrow. Hematopoietic cells share this bone cavity together with other cells, such as osteoblasts, osteoclasts, macrophages, adipocytes, perivascular cells, mesenchymal stem cells, and endothelial cells. The communications among these different cells are mediated through various cytokines, growth factors, matrix proteins, and cell-cell adhesions. All these factors have been demonstrated to be HSC niche component as they are co-localized with HSCs and have the functional effects on HSC self-renewal and differentiation (Scadden, 2006).

1.1.2.1 The anatomic location of HSCs in bone marrow

A few of the HSCs can migrate through the endothelial cells of vessels, circulate in the blood, and travel to other hematopoietic organs or come back home (BM) after circulation (Wright et al., 2001). Under such complicated trafficking inside and outside BM niche, the location of quiescent HSCs and more activated HSCs inside the BM are investigated.

By using SLAM family markers, many mouse HSCs labeled as $CD150^{+}CD244^{+}CD48^{-}$ or $CD150^{+}CD48^{-}CD41^{-}$ (LT-HSC) are found located adjacent to sinusoidal vessels, and some HSCs are found in the endosteum area (Kiel et al., 2007; Kiel et al., 2005). However, *ex vivo* image tracing indicates that HSCs (labeled by GFP expression) are located in the endosteum region of bones in the mice after irradiation, and most of them are detected in the trabecular area (Xie et al., 2009). Furthermore, HSCs express calcium-sensing receptors, and are located in calcium-rich endosteum (Adams et al., 2006). Additionally, several studies reveal the existence of HSCs at both sinusoidal area and endosteum area (Lo Celso et al., 2009; Nilsson et al., 2001; Sugiyama et al., 2006). Transplanted HSCs are found to locate in the area that were rich in CXCL12 expressing cells (CAR cells) (Sipkins et al., 2005), and these CAR cells are found either close to endosteum area or around sinusoidal vessels in BM (Lo Celso et al., 2009; Sugiyama et al., 2006). However, the HSCs located in the osteoblast niche of the endosteal region are more quiescent than that in the vascular niche (Kunisaki et al., 2013; Lo Celso et al., 2009; Nilsson et al., 2001).

1.1.2.2 BM cellular niche in the regulation of HSCs

1.1.2.2.1 Osteoblasts

Osteoblasts, the precursors of osteocytes, account for a very small amount (around 5%) within total bone cells (Capulli et al., 2014). Together with osteoclasts, osteoblasts participate in bone constructions to and contribute to the maintenance of HSC niche (Capulli et al., 2014). Through secreting collagen (e.g., collagen type 1) and non-collagen (such as

osteocalcin, osteopontin, and osteonectin) proteins, osteoblasts generate osteoid, which can be further mineralized to form osteocytes (Anderson, 2003).

Osteoblasts are considered as one of important BM niche components in the regulation of hematopoiesis (**Figure 2**) (Calvi et al., 2003; Zhang et al., 2003). Cell-cell attachments are observed between LT-HSCs and osteoblasts at the surface of the trabecular area in mouse bone (Zhang et al., 2003). The number of HSCs is positively correlated with the number of osteoblasts (Zhang et al., 2003). Moreover, osteoblasts express Notch ligand, jagged 1, and can promote BM HSC expansions through activated Notch signaling (Calvi et al., 2003). Meanwhile, the Angiopoietin-1 (Ang-1) expressed by osteoblasts can bind to its receptor Tie2 on HSCs, and therefore maintains the BM HSCs at quiescent status (Arai et al., 2004). Several BM niche factors are essential for hematopoiesis such as CXC chemokine ligand 12 (CXCL12) (Ding and Morrison, 2013; Greenbaum et al., 2013) and stem cell factor (SCF) (Czechowicz et al., 2007). CXCL12 is partially produced by osteoblasts, where the deletion of CXCL12 results in a mobilization of HSCs into circulation and reduced lymphoid progenitors (Greenbaum et al., 2013). On the other hand, osteoblasts support the lymphoid differentiation of HSCs *via* secreting interleukin (IL)-7 and cell-cell attachments (Zhu et al., 2007) or activating $G_s\alpha$ signaling (Wu et al., 2008). All together, these studies indicate that osteoblasts contribute to the formation of BM HSC niche, thereby regulate the expansion and quiescence of BM HSCs, and balance myeloid and lymphoid differentiations during hematopoiesis.

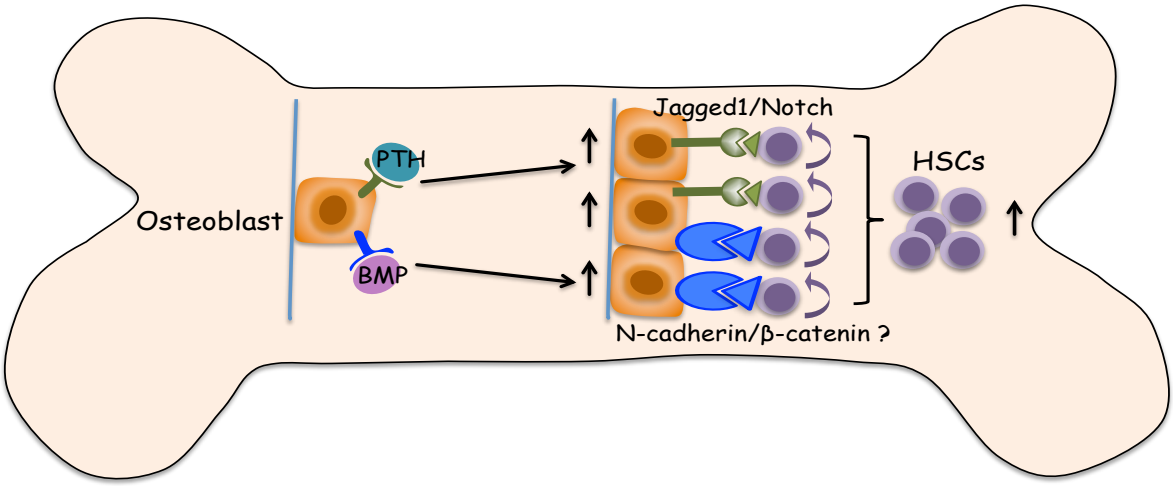

Figure 2. Osteoblasts control the BM niche size and regulate BM HSCs. Activated bone morphogenetic protein (BMP) signaling in osteoblasts increases the BM niche size followed by an expansion of BM HSCs. Activated parathyroid hormone (PTH) signaling enhances trabecular bone formations and elevates the level of Notch ligand, jagged 1, in osteoblasts. The activated Notch signaling in HSCs mediated by Notch and jagged 1 promotes BM HSC expansion.

Several studies have shown that impaired osteoblasts induce abnormal hematopoiesis in BM using different mouse models (**Figure 3**) (Visnjic et al., 2001; Visnjic et al., 2004; Zhu et al., 2007). By using the Col2.3Δtk transgenic mouse model, it has been shown the declined bone mass along with a dramatic reduction of BM B-lymphoid progenitors, HSCs, and total BM cellularity (Visnjic et al., 2004; Zhu et al., 2007). Meanwhile, extramedullary

hematopoiesis is noticed in the spleen and liver, which further confirms the impaired hematopoiesis in the mouse BM (Visnjic et al., 2004). Notably, the osteoblasts can be restored by withdrawing ganciclovir, followed by a recovery of BM hematopoiesis and reduced extramedullary hematopoiesis in the mice (Visnjic et al., 2004). Wnt signaling in BM niche is critical for maintaining BM HSC quiescence (Fleming et al., 2008; Schaniel et al., 2011). The inactivated Wnt signaling (overexpression of Wnt paninhibitor Dkk1 or Wif1) in Col2.3-labeled osteoblasts leads to the enhanced cell cycling of HSCs at the expense of quiescent HSCs (Fleming et al., 2008; Schaniel et al., 2011). The importance of osteoblasts to the BM niche is not only restricted at the adult stage, but also at the fetal stage. After the depletion of osterix^{+} osteoblasts in the mice at fetal stage, the BM hematopoietic cells present enhanced proliferations (Coskun et al., 2014). Although these BM HSPCs can form CFU-Cs *in vitro*, they lose their BM homing capacity and can not engraft in the recipient BM after transplantation (Coskun et al., 2014). Together, these studies provide evidences of osteoblasts as an important element for regulating HSC maintenance.

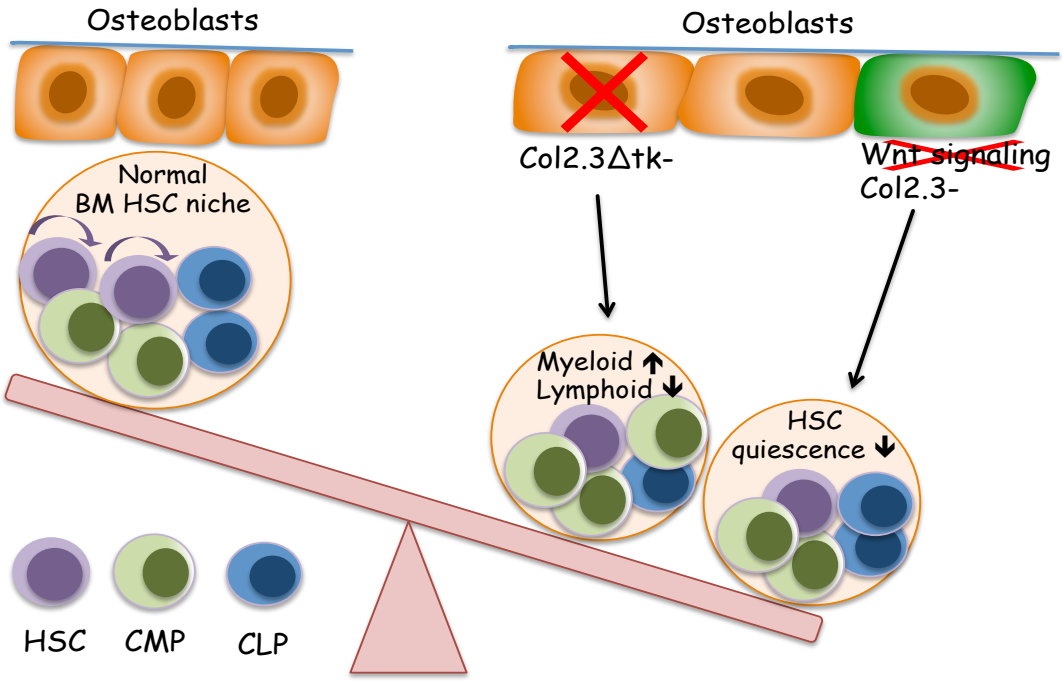

Figure 3. Abnormal BM hematopoiesis induced by impaired osteoblasts. The depletion of osteoblasts in Col2.3Δtk mice leads to reduced BM HSCs and B-lymphoid progenitors. The inactivated Wnt signaling in osteoblasts leads to a non-HSC autonomous decline of Wnt signaling in the HSCs, followed by the enhanced cell cycling of HSCs at the expense of the quiescent HSCs.

1.1.2.2.2 Endothelial cells

Endothelial cells are the surface layer of blood and lymphatic vessels, and directly in contact with blood or lymph. CD31 or Platelet/Endothelial Cell Adhesion Molecule 1 (PECAM-1) is a specific cell-surface membrane glycoprotein expressed on endothelial cells (Newman, 1994). During embryonic development, endothelial cells and hematopoietic cells share the same precursor, so called hemangioblasts, which express tyrosine kinase receptor fetal liver

kinase 1 (Flk1) (Choi et al., 1998; Shalaby et al., 1995). Mice without Flk1 at embryonic stage present dramatically declined hematopoiesis and vasculogenesis (Shalaby et al., 1995).

The relationship between endothelial and hematopoietic cells is not simply the connection by their common precursor, but also the crucial regulation of hematopoiesis by endothelial cells. *In vitro* co-culture experiments have shown that endothelial cells can recover the colony forming capacity of irradiated HSCs (Chute et al., 2004; Muramoto et al., 2006; Rafii et al., 1995). Endothelial cells can be isolated from different tissues, such as brain, heart, lung, liver, and kidney (Li et al., 2004). Among the diverse tissue-derived endothelial cells, the mouse/human brain-derived endothelial cells support hematopoiesis better than others through cell-cell adhesion and/or secreting soluble factors (Chute et al., 2005; Chute et al., 2007; Davis et al., 1995; Rafii et al., 1994). These studies provide the *in vitro* evidence of endothelial cells in supporting HSCs, and arouse the curiosity of the *in vivo* roles of endothelial cells in the regulation of hematopoiesis.

Using the approaches of real-time imaging, micro-CT, immunofluorescence imaging, and FACS, HSCs have been identified in vessel-enriched BM sinusoidal and endosteum areas (Ellis et al., 2011; Kiel et al., 2005; Xie et al., 2009). Taking advantage of various mouse models, the *in vivo* functions of endothelial cell in BM niche are further studied. The cytokine receptor subunit, gp130, is important in mediating the signals from the cytokines of IL6 family (Taga and Kishimoto, 1997). The abandon of gp130 in both hematopoietic and endothelial cells leads to the loss of LT-HSCs and extramedullary hematopoiesis in mice, which can not be restored after transplantation of normal BM cells (Yao et al., 2005). However, HSCs deficient with gp130 generate normal hematopoiesis after transplanting into wild type mice, indicating the gp130 expression in the endothelial cells is critical for maintaining normal hematopoiesis (**Figure 4**) (Yao et al., 2005). In addition, SCF (or KITL) and CXCL12 are known as critical BM niche factors (Ara et al., 2003; Broudy, 1997; Czechowicz et al., 2007; Sugiyama et al., 2006; Tzeng et al., 2011). The specific depletion of SCF in endothelial cells in the "*Tie2-cre;Scf*$^{fl/fl}$" mouse model leads to a loss of BM HSCs (Ding et al., 2012). The particular depletion of CXCL12 in endothelial cells in the "*Tie2-cre;Cxcl12* $^{fl/fl}$" mice also results in reduced BM HSCs (Ding and Morrison, 2013). These studies suggest that endothelial cells are crucial BM cellular niche components for maintaining the BM HSC pool *in vivo*.

Irradiation and chemotherapy lead to BM suppression with damaged hematopoietic cells and vessels, although the effects of chemotherapy and sub-lethally irradiation are less serious than that of lethally irradiation (Hooper et al., 2009). The regeneration of BM hematopoiesis after irradiation is regulated by BM endothelial cells (Hooper et al., 2009; Salter et al., 2009; Winkler et al., 2012). *In vivo* transplantation of endothelial progenitor cells enhanced mouse BM recovery after irradiation (Salter et al., 2009). However, the application of an anti-VE-cadherin antibody inhibits the vessel formation, and postpones the hematopoiesis and vasculogenesis in the mice (Salter et al., 2009). Apart from BM endothelial cells, the *in vivo* infusion of mouse brain-derived endothelial cells alone leads to a

better survival of the lethally irradiated mice than those infused with fetal blood-derived endothelial cells (Chute et al., 2007). Interestingly, the expression of an HSC related growth factor, pleiotrophin, is detected with a 25-fold higher expression in human brain-derived endothelial cells than in other tissue derived endothelial cells (Himburg et al., 2010). *In vivo* administration of pleiotrophin to the irradiated mice induce an enhanced BM hematopoiesis recovery through the Phosphoinositide 3 kinase (PI3K) signal pathway (Himburg et al., 2010).

A series of angiocrine factors produced by endothelial cells are involved in the vascular HSC niche regulation. E-selectin, an adhesion molecule, is specifically detected in BM endothelial cells and is essential for promoting HSC proliferations in mice (Winkler et al., 2012). In the *Sele*$^{-/-}$ mice, where the E-selectin is specifically depleted, the BM HSCs became more quiescent with an increased self-renewal capacity (**Figure 4**) (Winkler et al., 2012). Interestingly, endothelial cells differentially regulate the HSCs/HPCs with different activities through different signal pathways (**Figure 4**). For example, endothelial cells maintain HSC self-renewal through the expression of Notch ligand (Butler et al., 2010) or increased angiocrine factors activated by Akt-mTOR signaling in the endothelial cells (Kobayashi et al., 2010); while endothelial cells with a co-activation of mitogen-activated protein kinases (MAPK) signaling and Akt-mTOR signaling maintain the HSPCs which are prone to lineage differentiation (**Figure 4**) (Kobayashi et al., 2010).

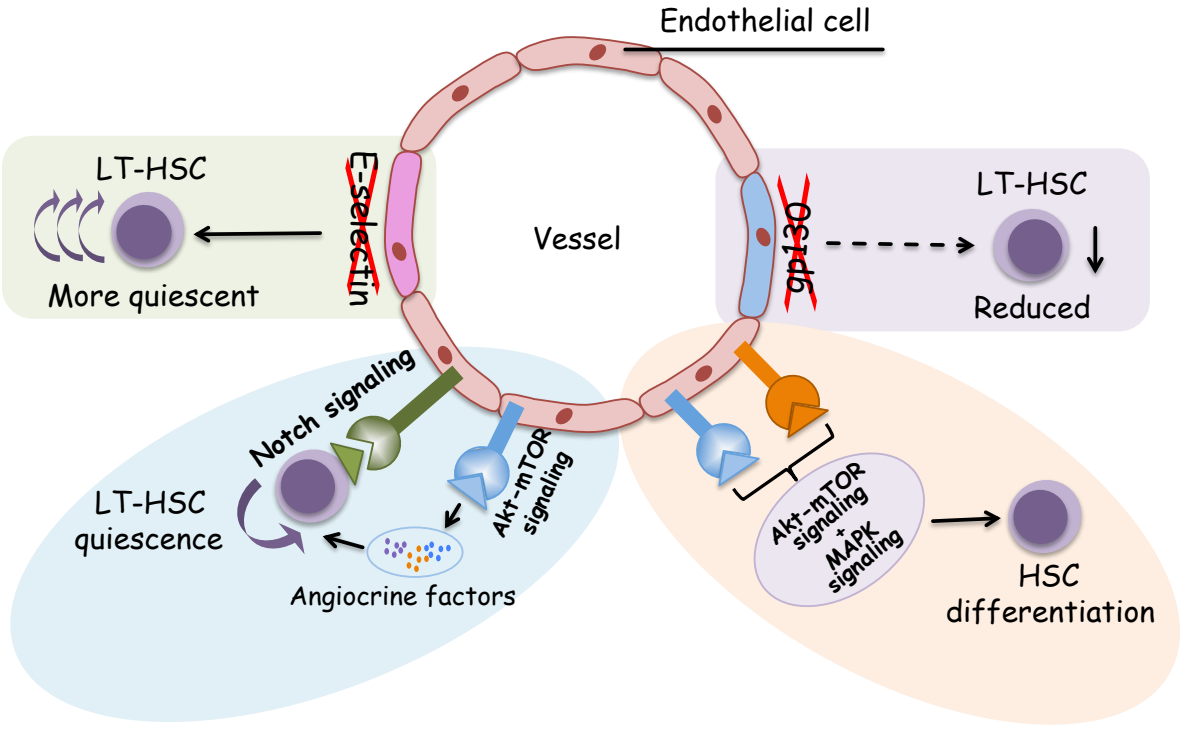

Figure 4. Regulations of HSC quiescence and differentiation from Endothelial cells. The gp130 expression in endothelial cells is critical for maintaining LT-HSCs. The expression of E-selectin in endothelial cells is essential for promoting HSC proliferations. Endothelial cells maintain HSC self-renewal through the expression of Notch ligand or increased angiocrine factors activated by Akt-mTOR signaling in the endothelial cells. Endothelial cells with a co-activation of MAPK signaling and Akt-mTOR signaling maintain the HSPCs which are prone to lineage differentiation.

Vascular endothelial growth factor (VEGF) is produced by various cell types, such as macrophages (Sunderkotter et al., 1994), platelets (Verheul et al., 1997), cancer cells (Boocock et al., 1995), and endothelial cells (Lee et al., 2007). VEGF binds to its receptor VEGFR1 and VEGFR2 that expressed in endothelial cells to promote angiogenesis (Carmeliet et al., 1996; Ferrara et al., 1996; Mustonen and Alitalo, 1995). Mice with only one allele of *Vegf* fail to survival due to the impaired vessel formation at the embryonic stage (Carmeliet et al., 1996; Ferrara et al., 1996). By using (*VE-Cadherin-Cre* x *Vegf*$^{lox/lox}$) mouse model, the depletion of *Vegf* in the endothelial cells leads to impaired angiogenesis, followed by an early death of the mice (Lee et al., 2007). However, the total level of VEGF protein is not much altered in these mice, indicating that the autocrine VEGF signaling in the endothelial cells results in the mouse death (Lee et al., 2007). Furthermore, endothelial cells express VEGFR2 and participate in the regeneration of irradiation-induced BM suppression in the mice. The conditional depletion of VEGFR2 has little effect on mouse hematopoiesis at steady state, but impairs BM hematopoietic reconstitution after irradiation (Hooper et al., 2009). These studies do not show direct regulations of BM hematopoiesis by VEGF and VEGFR2, but might implying an indirectly effect on hematopoiesis mediated by endothelial cells and angiogenesis.

It is believed that endothelial cells located at sinusoidal vessels and arteries play different roles in regulating hematopoiesis (**Figure 5**). With a common expression of VEGFR2 on the endothelial cells both sinusoids and arteries, sinusoidal endothelial cells are further marked by VEGFR3, whereas arterial endothelial cells are further labeled by SCA1 (Hooper et al., 2009). The arteries are less permeable than sinusoids, located close to endosteum area, and present a low level of reactive oxygen species (ROS) for maintaining HSC quiescence (Itkin et al., 2016; Spencer et al., 2014). The sinusoids are mainly located at central marrow with a relatively higher ROS level, and form a special site for hematopoietic cells entering into circulation and coming back to BM (Itkin et al., 2016). Furthermore, the arterial endothelial cells are sheathed by Nerual/glial antigen 2 (NG2) pericytes which enhance HSC quiescence (Kunisaki et al., 2013). In addition, type H endothelial cells with high expressions of CD31 and Endomucin (CD31^{hi}Emcn^{hi}) are located in endosteum and trabecular area supporting osteogenesis (Kusumbe et al., 2014), which are indirectly involved in the hematopoiesis regulation. Take together, endothelial cells through direct cell-cell contact, cytokines, and angiocrine factors, as well as indirect pathways contribute to the BM vascular niche for maintaining hematopoiesis.

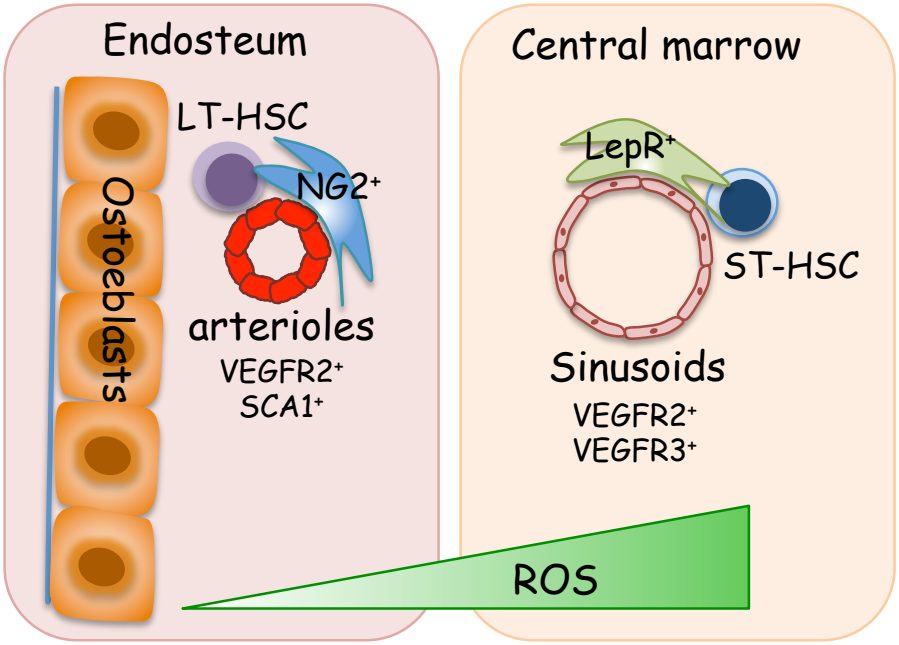

Figure 5. BM HSC vascular niche in endosteum area and central marrow. Sinusoidal endothelial cells are marked as $VEGFR2^{+}VEGFR3^{+}$, whereas arterial endothelial cells are labeled by $VEGFR2^{+}SCA1^{+}$. The arteries are located close to endosteum area, and present a low ROS level for maintaining HSC quiescence. The sinusoids are mainly located at central marrow with a relatively higher ROS level, and form a special site for hematopoietic cells trafficking. Arterial endothelial cells are sheathed by NG2 pericytes, which enhance HSC quiescence, whereas sinusoidal endothelial cells are surrounded by $LepR^{+}$ cells.

1.1.2.2.3 Adipocytes

In adult human bone, BM hematopoietic cells are mainly located in metaphysis area, whereas BM adipocytes occupy most of space in diaphysis. These BM adipocytes, also known as yellow marrow, do not exist in diaphysis at newborn stage (Blebea et al., 2007). These adipocytes can be produced by BM stromal cells through activated leptin/lepR signaling (Yue et al., 2016). Perilipin, a lipid marker for adipocytes (Greenberg et al., 1991), has been applied in immunohistochemistry staining for the identification of adipocytes in tissue. BM adipocytes are not simply occupying the bone cavity, but play crucial roles in the regulation of hematopoiesis.

It has been shown that adipocytes cannot maintain the quiescence of HSCs, but promote HSC differentiation in co-cultivation (Corre et al., 2004). The cell-cell interactions between adipocytes and HSPCs can up-regulate the expression of neuropilin-1 on the adipocytes, leading to a reduction of granulocyte colony-stimulating factor (G-CSF) and a resultant inhibition of granulopoiesis (Belaid-Choucair et al., 2008). These studies indicate a negative regulation of hematopoiesis by BM adipocytes.

In mice, BM HSCs have been shown to mainly locate in the adipocyte-free thoracic vertebrae, whereas a small number of HSCs locate in the caudal vertebrae where contains abundant of adipocytes (Naveiras et al., 2009). By using a fatless transgenic mouse model, it

has been shown an enhanced engraftment of HSCs in the fatless mouse BM along with an increased trabecular bone formation after BM transplantation (Moitra et al., 1998; Naveiras et al., 2009). Similarly, the inhibition of adipogenesis also increases HSC engraftment in irradiated mouse BM (Naveiras et al., 2009). Together, these studies suggest that adipocytes are an important BM niche component and negatively regulate the hematopoiesis in the BM.

1.1.2.2.4 Megakaryocytes

Megakaryocytes are hematopoietic lineage cells, which are identified as $Lin^{-}cKit^{+}Sca1^{-}CD150^{+}CD41^{+}$ in mouse BM (Nakorn et al., 2003; Pronk et al., 2007). In addition to the production of platelets, megakaryocytes can also regulate HSC activities. Platelet factor-4 (PF-4), a member of the CXC chemokine family (CXCL4) (Slungaard, 2005), is mainly synthesized and stored in megakaryocytes, and released into BM together with platelets (Lasagni et al., 2007; Slungaard, 2005). PF-4 released by megakaryocytes maintains HSC quiescence through the inhibition of IL8-induced HSC activation and the enhanced adhesion of human HSCs to stromal cells (Dudek et al., 2003). Additionally, megakaryocytes have been shown to promote the proliferation of HSCs through insulin-like growth factor 1 (IGF-1) signaling (Heazlewood et al., 2013) (**Figure 6**).

Megakaryocytes attach to the vascular cells of the BM sinusoids (Avecilla et al., 2004; Junt et al., 2007) and are adjacent to HSCs (Bruns et al., 2014; Heazlewood et al., 2013; Zhao et al., 2014). Thrombopoietin (THPO) has been known to be critical for the maintenance of HSCs (Qian et al., 2007; Yoshihara et al., 2007). It has been found that the depletion of megakaryocytes in mice leads to a reduction of THPO in BM, followed by a mobilization of HSCs into circulation (Nakamura-Ishizu et al., 2014). Moreover, megakaryocytes can keep the quiescence of BM HSCs through the production of CXCL4 (Bruns et al., 2014). In addition, megakaryocyte-mediated transforming growth factor β 1 (TGFβ1) signaling is also known to be critical for maintaining HSC quiescence (Zhao et al., 2014). Mice with the depletion of megakaryocytes present a reduction of TGFβ1 in the BM, followed by a proliferation of BM HSCs (Zhao et al., 2014). A similar phenomenon is observed in the TGFβ1 knockout mice, where the quiescence of the HSCs can be restored after the treatment of TGFβ1 (Zhao et al., 2014). Together, these studies suggest that megakaryocytes are a critical BM cellular niche component and participate in the maintenance of BM HSC quiescence (**Figure 6**).

However, under stress conditions, such as irradiation or chemotherapy, where BM hematopoietic cells and niche are damaged, megakaryocytes have been shown to actively support the hematopoiesis. It have been found that megakaryocytes migrate to the endosteal niche by activated THPO signaling to assist osteogenesis in irradiated mouse (Olson et al., 2013). Furthermore, megakaryocytes promote the regeneration of endosteal osteoblasts in the irradiated mice by producing platelet-derived growth factor-BB (PDGF-BB) (Olson et al., 2013). The interruption of THPO signaling by blocking THPO receptor (c-MPL) on megakaryocytes can reduce the engraftment of HSCs in the BM; while the application of THPO improves the BM hematopoiesis in the irradiated mice (Olson et al., 2013). Another

cytokine, fibroblast growth factor1 (FGF1), mainly produced by megakaryocytes, has been shown to support HSPC expansion *in vitro* (Faloon et al., 2000). Under chemotherapy-induced stress condition, megakaryocytes can accelerate the proliferation of BM HSCs through the activated FGF1 signaling, which overwhelms the TGFβ1 signaling-induced inhibition of HSCs (Zhao et al., 2014; Zhao et al., 2012). All together, these studies suggest that megakaryocytes play critical roles in maintaining of BM HSC quiescence under homeostatic condition, and promoting the recovery of BM HSC niche under stress condition (**Figure 6**).

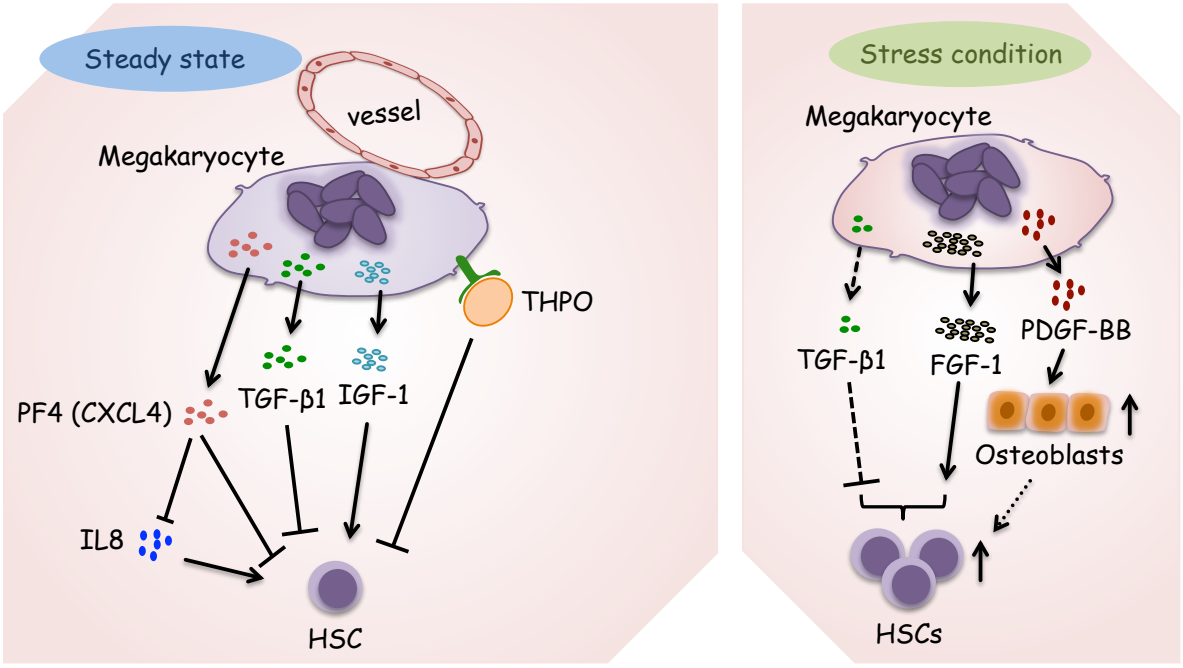

Figure 6. Regulations of HSCs by Megakaryocytes under the steady state and stress condition. At steady state, megakaryocytes maintain HSC quiescence through CXCL4 and TGF-β1 signal pathways, and promote HSC expansion through IGF-1 signaling. THPO preserves HSC quiescence at steady state and assists the migration of megakaryocytes to the endosteal niche to support HSC niche regeneration in irradiated mice. Megakaryocytes can produce PDGF-BB and FGF-1 to facilitate the recovery of the BM HSC niche under stress conditions.

1.1.2.2.5 Mesenchymal stem cells (MSCs)

1.1.2.2.5.1 Criteria of defining MSCs

The concept of BM HSC niche was proposed by Schofield in 1978. Prockop later confirmed that BM mesenchymal stromal cells could regenerate non-hematopoietic tissue for wound healing (Prockop, 1997). These mesenchymal stromal cells are selected by the plastic adherence in cultivation, and are further used for tissue regenerations *in vivo*. The ambiguous "MSCs" is the acronym for both mesenchymal stromal cells and mesenchymal stem cells. However, not all the adherent stromal cells present stem cell characteristics, including self-renewal and multi-lineage differentiations (Horwitz and Keating, 2000). According to the standard from the International Society for Cellular Therapy (ISCT), "MSCs" only refers to the multipotent mesenchymal stromal cells with stem cell characteristics (Horwitz et al.,

2005). Four minimal criteria of the definition of human mesenchymal stromal cells have been suggested (Dominici et al., 2006). First, MSCs are adherent stromal cells obtained from the standard culture selection. Second, MSCs express mesenchymal stem cell markers, such as CD150, CD90, and CD73. Third, MSCs do not express any hematopoietic markers, including CD45, CD14, CD34, CD11b, CD19, CD79α, and HLA-DR molecules. Finally, MSCs possess multi-lineage differentiation capacities, including adipogenic, osteogenic, and chondrogenic differentiations *in vitro*.

1.1.2.2.5.2 Cell surface markers of human BM MSCs

Due to the heterogeneity of MSCs, several cell surface markers have been applied to better identify the BM MSCs. The low affinity nerve growth factor receptor, also known as CD271, expresses in human BM stromal cells (Cattoretti et al., 1993). These $CD271^+$ stromal cells mingle with hematopoietic cells, attach to the surface of sinusoids, and form a supportive scaffold for the BM cells (Cattoretti et al., 1993). The colony forming ability of the $CD271^+$ stromal cells has been confirmed by colony-forming unit fibroblast (CFU-F) assay with a frequency of 1584 out of 1 million input cells, whereas no colony is detected in $CD271^-$ fraction (Quirici et al., 2002). Moreover, $CD271^+$ stromal cells possess multi-lineage differentiation potentials, which can generate adipocytes, osteoblasts, and chondrocytes *in vitro* (Jones et al., 2006). In addition, $CD271^+$ stromal cells express *fatty acid binding protein 4* (*FABP4*), *SP7*, *CXCL12*, and *angiopoietin like 1* (*ANGPTL1*), suggesting the multi-potency and HSC supportive potentials of $CD271^+$ stromal cells *in vivo* (Churchman et al., 2012). Together, these evidences indicate that CD271 is a surface marker for human MSCs.

Given the fact that not every $CD271^+$ stromal cell possesses stem cell characteristics, other markers are applied to identify human MSCs. The $STRO\text{-}1^+$glycophorin A^- BM cells have been considered as MSCs due to their capacities of CFU-F and multi-lineage differentiation *in vitro* (Simmons and Torok-Storb, 1991). Furthermore, CD105 (Endoglin) (Barry et al., 1999), CD49a (Integrin α1) (Deschaseaux and Charbord, 2000), CD90 (Thymocyte differentiation antigen 1, THY1) (Vogel et al., 2003), CD140B (Platelet-derived growth factor receptor beta, PDGFRB) (Vogel et al., 2003), CD166 (Stewart et al., 2003), CD63 (Zannettino et al., 2003), and CD73 (Boiret et al., 2005) have been also identified as human MSC markers. In addition, MSCs express CD146 (melanoma cell adhesion molecule, MCAM), which partially overlaps with CD105, CD49a, CD63, CD90, and CD140B antigens (Sacchetti et al., 2007). These $CD146^+$ cell clones can self-renew after transplanting into NOD scid gamma (NSG) mice, and generate human bone and adventitial cells in the mice (Sacchetti et al., 2007). However, these markers label very heterogeneous cell populations, indicating their limitations in the identification of MSCs.

To further purify BM MSCs, multicolor FACS has been employed. Combined with CD146 marker, the $CD271^+$ MSCs are subdivided into $CD271^+CD146^+$ and $CD271^+CD146^{-/low}$ cells (Tormin et al., 2011). Both of them form CFU-Fs *in vitro*, but the $CD271^+CD146^+$ MSCs sit in the perivascular area, whereas the $CD271^+CD146^{-/low}$ MSCs restrict in the endotheum region (Tormin et al., 2011). These results indicate that the phenotype of BM

MSCs varies between the endosteal niche and perivascular niche. The BM MSCs defined by the combination of $CD271^+CD90^+CD106^+$ display a high CFU-F capacity with the frequency of one colony out of three input cells (Mabuchi et al., 2013). Moreover, the BM MSCs identified by $CD45^-CD31^-CD71^-CD146^+CD105^+nestin^+$ can grow as non-adherent mesenspheres, differentiate into multi-lineage, and promote HSC expansion (Isern et al., 2013). In addition, $CD140A^+CD51^+$ stromal cells are enriched with MSCs in human fetal BM, which overlap with $Nestin^+$ cells (Pinho et al., 2013). These human fetal $CD140A^+CD51^+$ MSCs can also form non-adherent mesenspheres, differentiate into adipocytes, osteoblasts, and chondrocytes, and support HSC expansion (Pinho et al., 2013) (**Table 1**).

However, the phenotype of MSCs changes in different conditions, such as *in vitro* adhesive cultivation, the oxygen level of the culture condition, and growth factors in the culture media. First, freshly isolated MSCs do not express CD44, but acquire CD44 expression when they are in an *in-vitro* cultivation (Qian et al., 2012). Second, the CD146 expression of BM MSCs is higher in the normoxic culture condition than the hypoxic condition (Tormin et al., 2011). Third, $CD271^+$ MSCs cultured in the basic fibroblast growth factor (bFGF) free media display the higher expression of CD271 than those cultured in the media containing bFGF (Quirici et al., 2002).

Table 1. Identified MSC markers of human BM.

MSC markers (Human)	Positive	CD271, CD146, STRO1, CD49a, CD90, CD140B, CD105, CD166, CD63, and CD73	
	Negative	CD44	
Combination (Within $CD45^-CD235^-CD31^-$)		Tissue	Reference
$CD271^+CD146^+$		Adult BM	Tormin et al., 2011
$CD271^+CD90^+CD106^+$		Adult BM	Mabuchi et al., 2013
$CD71^-CD146^+CD105^+Nestin^+$		Adult BM	Isern et al., 2013
$CD140A^+CD51^+$		Fetal BM	Pinho et al., 2013

1.1.2.2.5.3 Cell surface markers of mouse BM MSCs

Hematopoietic and mesenchymal lineages are two distinct BM cell populations, although they express many common cell surface markers, such as CD49e, CD105, Sca1, Tie-2, and CD34 (Koide et al., 2007). Based on combined hematopoietic lineage markers and several MSC markers, the phenotype and function of murine MSCs are further identified (**Figure 7**).

BM $CD140A^+CD51^+$ stromal cells not only present human fetal BM MSCs, but also mouse MSCs (Pinho et al., 2013). Using a mouse model where $Nestin^+$ cells are labeled by green fluorescent protein (GFP), about 75% of $CD140A^+CD51^+$ MSCs are $Nestin^+$, whereas

around 60% of total Nestin$^+$ cells are CD140A$^+$CD51$^+$ (Pinho et al., 2013). These BM CD140A$^+$CD51$^+$ MSCs can form CFU-Fs and non-adherent mesenspheres, self-renew, and differentiate into adipocytes, osteoblasts, and chondrocytes *in vitro* (Pinho et al., 2013). Importantly, HSC niche genes, such as *Scf*, *Cxcl12*, *vascular cell adhesion protein 1* (*Vcam1*), *Angpt1*, and *osteopontin* (*Opn*), are highly expressed in the BM CD140A$^+$CD51$^+$ MSCs, indicating the HSC supportive function of the CD140A$^+$CD51$^+$ MSCs (Pinho et al., 2013). Another combination of CD140A and Sca1 marks mouse BM MSCs as CD45$^-$ TER119$^-$CD31$^-$CD140A$^+$Sca1$^+$ (Morikawa et al., 2009a; Morikawa et al., 2009b). The CD140A$^+$Sca1$^+$ MSCs have a high colony forming capacity with the frequency of 1 colony out of 40 input cells (Morikawa et al., 2009a). Compared with CD140A/Sca1 single positive and double negative stromal cells, the CD140A$^+$Sca1$^+$ MSCs exhibit much stronger multi-lineage differentiation potentials *in vitro* (Morikawa et al., 2009a). HSC niche factors, such as *Angpt1* and *Cxcl12*, are highly expressed in the CD140A$^+$Sca1$^+$ MSCs, which can support HSC reconstitutions in lethally irradiated mice (Morikawa et al., 2009a). These results suggest that CD140A is a specific mouse MSC marker, and that the combination of CD140A and CD51 or Sca1 can better characterize mouse MSCs.

Apart from these combinations of MSC positive marker, it is worth mentioning a negative marker enriched for MSCs, CD44, which does not express on freshly isolated MSCs (Qian et al., 2012). Notably, CFU-Fs are enriched in the CD44$^-$ stromal cells, but not in the CD44$^+$ stromal cells (Qian et al., 2012). Accordantly, the MSC markers of CD140A, CD51, and Sca1 are only detected in the CD44$^-$ stromal cells (Qian et al., 2012). Moreover, CD44$^-$ stromal cells exhibit much higher expression of niche associated genes than CD44$^+$ stromal cells, such as *Nestin*, *collagen type I alpha 1* (*Col1a1*), *Angptl1*, *insulin-like growth factor 1* (*Igf1*), *Fibromodulin* (*Fmod*), and *nephroblastoma overexpressed* (*Nov*) (Qian et al., 2012). This study suggested that naïve MSCs do not express CD44 antigen, and CD44 can be applied as a negative marker for the MSC enrichment.

Other stromal cell markers, such as CD105 and CD140B, are also tested for identifying MSCs, but the labeled cells are very heterogeneous and restricted to mesenchymal lineages. For example, CD105-expressing stromal cells are restricted to an osteo-lineage-biased MSC population in mouse BM (Chan et al., 2013). CD140B is expressed in culture-selected MSCs and freshly isolated non-hematopoietic mouse cells, indicating CD140B is a potential marker for mesenchymal lineage cells (Koide et al., 2007).

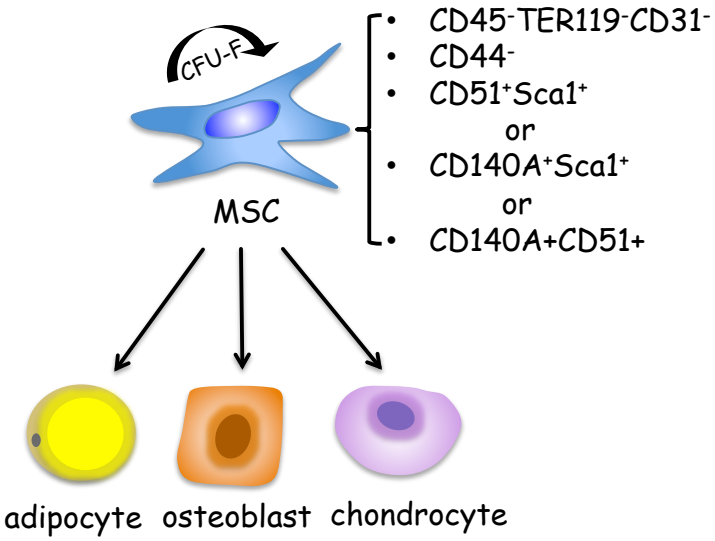

Figure 7. Phenotypic and functional identification of mouse BM MSCs. Naïve mouse BM MSCs do not express the hematopoietic marker CD45 or TER119, and are negative for CD31 and CD44, but positive for CD51, CD140A, and Sca1. MSCs can form CFU-Fs *in vitro* and differentiate into adipocytes, osteoblasts, and chondrocytes.

1.1.2.2.5.4 BM MSCs/MSPCs defined by using genetic mouse models

By taking the advantage of genetically modified mouse models, MSCs labeled by specific gene markers and can be tracked for their locations and functions *in vivo*. Here I summarize several identified MSCs using different genetic mouse models (**Figure 8**).

❖ **LepR$^+$ MSCs**

Leptin Receptor (LepR) labeled cells attach to sinusoids and arteries and are identified as perivascular cells with MSC characteristics in adult mice (Zhou et al., 2014). LepR$^+$ MSCs are a rare cell population in mouse BM with an average frequency of 0.22% of BM CD45$^-$ TER119$^-$CD31$^-$ stromal cells (Zhou et al., 2014). Phenotypically, more than 98% LepR$^+$ MSCs express CD140A or CD51 (Zhou et al., 2014), and 70% LepR$^+$ MSCs express both CD140A and CD51 (Pinho et al., 2013). Up to 94% of the *in vitro* colonies formed by BM mononuclear cells are contributed by LepR$^+$ MSCs, indicating the high CFU-F capacity of the LepR$^+$ MSCs (Zhou et al., 2014). Moreover, LepR$^+$ MSCs are quiescent at steady state, but give rise to adipocytes, osteoblasts, and chondrocytes *in vivo* after irradiation or bone injury (Zhou et al., 2014). However, LepR$^+$ MSCs lose LepR expression after differentiating into mature osteolineages, suggesting that LepR$^+$ MSCs generate LepR$^-$ lineages *in vivo* (Mizoguchi et al., 2014). Additionally LepR$^+$ MSCs express the BM niche maintenance genes, such as Scf and CXCL12 (Zhou et al., 2014). The specific deletion of *Cxcl12* from LepR$^+$ MSCs leads to the mobilization of BM HSPCs into the spleen and blood (Ding and Morrison, 2013). The conditional depletion of SCF from LepR$^+$ MSCs leads to a dramatic reduction of HSCs in mouse BM, suggesting the crucial role of SCF from LepR$^+$ MSCs in maintaining HSCs (Ding et al., 2012). Together, these studies suggest that LepR$^+$ MSCs are

not only the major source for different BM niche cells, but are also important for the maintenance of HSCs in BM niche.

❖ Ebf2^{+} MSCs

Early B cell factor 2 (Ebf2) is a transcription factor of the Ebf family (Garel et al., 1997). In contrast to the expression of Ebf1 in B lymphocytes, Ebf2 is not detectable in the hematopoietic cells of adult mice (Kieslinger et al., 2010; Qian et al., 2013). However, Ebf2 is expressed in osteoprogenitors (Kieslinger et al., 2005; Kieslinger et al., 2010), adipoprogenitiors (Rajakumari et al., 2013; Wang et al., 2014), neurons (Giacomini et al., 2011; Wang et al., 2004; Yang et al., 2015) and BM niche cells (Qian et al., 2013). By taking advantages of Tg(*Ebf2-GFP*) transgenic mouse model, Ebf2 is identified as a BM MSC marker in mice (Qian et al., 2013). BM Ebf2^{+} MSCs are mainly located in the endosteal area and possess multi-lineage differentiation capacities *in vivo* and *in vitro* (Qian et al., 2013). Phenotypically, Ebf2^{+} MSCs express MSC-related cell surface markers, such as SCA1, CD51, CD140A, and CD90, but do not express CD44 (Qian et al., 2012). Notably, Ebf2^{+} MSCs exhibit a high capacity of CFU-Fs with a frequency of 1 colony out of 6 input cells, which is higher than that of CD140A^{+}SCA1^{+} MSCs (1 out of 40 input cells) (Morikawa et al., 2009a; Qian et al., 2013). Furthermore, BM niche genes are enriched in the BM Ebf2^{+} MSCs, such as *runt-related transcription factor 2* (*Runx2*), *Angptl1*, *Nov*, *Fmod*, *Nestin*, and *N-cadherin*, indicating HSC supportive functions of the Ebf2^{+} MSCs (Qian et al., 2013). Additionally, the mice without Ebf2 (Ebf2$^{-/-}$) show reduced HSPC number and declined lymphopoiesis (Kieslinger et al., 2010). Nevertheless, the BM cells of the Ebf2$^{-/-}$ mice generate normal hematopoiesis after transplanting into a normal BM niche, indicating the niche-dependent hematopoietic disorder in the Ebf2$^{-/-}$ mice (Kieslinger et al., 2010). On the other hand, Ebf2$^{-/-}$ stromal cells impair the function of normal HSCs in co-culture, implying a critical role of Ebf2 expression in the BM niche (Kieslinger et al., 2010). All together, these studies suggest that Ebf2^{+} MSCs are fulfill the MSC criteria and are essential niche cells for maintaining normal hematopoiesis in mouse BM.

❖ Nestin^{+} MSCs

By using the mouse model Tg(*Nestin-GFP*) for lineage tracing, Nesin^{+} cells are identified as the neural stem/progenitor cells in mouse brain (Mignone et al., 2004). In mouse BM, Nestin^{+} cells mark a specific cell population displaying MSC characteristics and locate closely to HSCs and vessels (Mendez-Ferrer et al., 2010). These Nestin^{+} MSCs can form CFU-Fs and non-adherent mesenspheres and differentiate into adipocytes, chondrocytes, and osteoblasts *in vitro* (Mendez-Ferrer et al., 2010). Based on the expression levels of GFP, Nestin^{+} MSCs are divided into Nestin^{+} GFP^{high} MSCs and Nestin^{+} GFP^{dim} MSCs (Kunisaki et al., 2013). Most of Nestin^{+} MSCs are labeled by dim GFP and close to sinusoids, whereas the Nestin^{+} GFP^{high} MSCs are much rare in numbers and locate in the periarteriolar area (Kunisaki et al., 2013). Several BM niche genes for maintaining HSC homeostasis are detected in Nestin^{+} MSCs, such as *Cxcl12*, *Kitl*, *Angpt1*, *Il7*, *Vcam1*, and *Spp1* (Mendez-Ferrer et al., 2010). The specific depletion of Nestin^{+} MSCs using the (*Nes-cre* ^{ER2};*iDTR*) mouse model, leads to

reduced BM HSPCs and increased spleen HSPCs (Mendez-Ferrer et al., 2010). These studies indicate that Nestin$^+$ MSCs are a critical BM cellular niche component for maintaining BM hematopoiesis.

Although *Cxcl12* is highly expressed in Nestin$^+$ MSCs, the depletion of *Cxcl12* from Nestin$^+$ MSCs using "*Nestin-Cre* x *Cxcl12*$^{fl/fl}$" mouse model has no effect on hematopoiesis (Balordi and Fishell, 2007; Ding and Morrison, 2013). Moreover, the depletion of *Scf* from Nestin$^+$ MSCs does not affect hematopoiesis in the mouse BM, but reduces the HSC frequency in the mouse spleen (Ding et al., 2012). These studies indicate that CXCL12 and SCF from the Nestin$^+$ MSCs are dispensable for the hematopoiesis in the mouse BM. However, SCF secreted by the Nestin$^+$ MSCs may be important for the hematopoiesis in the mouse spleen.

In one week postnatal mice, Nestin marks not only BM MSCs, but also other stromal cell types. More than 90% osteolineage cells labeled by osterix (Osx), Col1 (3.2kb), or osteocalcin (Ocn) express Nestin (Ono et al., 2014). Most of the Tie2$^+$ endothelial cells and the majority of the LepR$^+$ MSCs are also positive for Nestin (Ono et al., 2014). In addition, about 96% Nestin$^+$ cells express NG2 in adult mice (Asada et al., 2017). These evidences suggest that postnatal Nestin$^+$ cells are heterogeneous stromal populations, including osteoprogenitors, osteoblasts, endothelial cells, NG2 periarteriolar cells, and MSCs.

❖ Prx1$^+$ MSCs

Paired related homeobox gene 1 (Prx1) expressing cells are heterogeneous mesenchymal stromal cells and are involved in limb formations (Logan et al., 2002). About 95% of Prx1$^+$ cells express CD140A (Ding and Morrison, 2013), and around half of the PαS cells (CD140A$^+$Sca1$^+$ MSCs) are positive for Prx1 expression (Greenbaum et al., 2013). Prx1$^+$ cells have a high CFU-F capacity with an average frequency of 1 colony out of 10 input cells, and can differentiate into adipocytes and osteoblasts *in vitro* (Greenbaum et al., 2013). These Prx1$^+$ MSCs secrete CXCL12; and the conditional depletion of *Cxcl12* from Prx1$^+$ MSCs leads to the reduced HSCs and lymphoid progenitors in the mouse BM (Ding and Morrison, 2013; Greenbaum et al., 2013). Furthermore, the depletion of LepR from Prx1$^+$ MSCs results in an enhanced bone formation and declined adipocyte differentiation in mice (Yue et al., 2016). Collectively, these studies suggest that Prx1$^+$ MSCs are important for replenishing BM niche cells and maintaining HSC pools in the mouse BM.

❖ Mx1$^+$ MSCs

Myxovirus resistance-1 (Mx1) expressing cells within the mouse BM stromal cells are also characterized as MSCs (Park et al., 2012). About half of the CD105$^+$CD140A$^+$ stromal cells express Mx1, indicating an MSC phenotype of the Mx1$^+$ cells (Park et al., 2012). They can form CFU-Fs *in vitro*, with an average frequency of 1 colony out of 40 input cells (Park et al., 2012). Although Mx1$^+$ MSCs can differentiate into adipocytes, chondrocytes, and osteoblasts *in vitro*, they are restricted to osteolineages and contribute to bone fracture regenerations *in*

vivo (Park et al., 2012). Due to these features, mouse BM $Mx1^+$ MSCs are considered as osteo-restricted progenitors *in vivo*, although they fully fit the criteria of MSCs *in vitro*.

❖ **$Cxcl12^+$ MSPCs (CAR cells)**

The chemokine CXCL12 is also known as pre-B cell growth stimulating factor (PBSF) or stromal cell-derived factor 1 (SDF-1) (Nagasawa et al., 1996; Nagasawa et al., 1994). CXCL12 is produced by different BM stromal cells, such as MSCs, MPCs, endothelial cells, reticular cells, osteoblasts, and mature BM stromal cells (Sugiyama et al., 2006). CXCL12-abundant reticular cells (CAR cells) refer to the BM stromal cells with a high CXCL12 expression (Sugiyama et al., 2006). CAR cells are not embedded in the mineralized bone, but sporadically distributed in the BM cavity (Sugiyama et al., 2006). Phenotypically, BM CAR cells are positive for VCAM-1 (CD106), mostly positive for MSC marker CD51 and CD140A/B, low/negative for CD44, and undetectable for SCA1 (Omatsu et al., 2010). The freshly isolated BM CAR cells fail to form colonies *in vitro*, indicating that CAR cells have little self-renewal capacity (Omatsu et al., 2010). CAR cells express differentiation genes, such as *Runx2*, *Osterix*, and, *peroxisome proliferator-activated receptor gamma* (*PPARγ*), which are related to the osteogenic and aidpogenic differentiations in special culture media (Omatsu et al., 2010). Moreover, CAR cells involve in the maintenance of BM homeostasis through CXCL12-CXCR4 signaling and CAR cell-HSC adhesions (Sugiyama et al., 2006). In addition to the secretion of CXCL12, CAR cells also produce a high level of SCF (Omatsu et al., 2010), which is critical for lymphopoiesis and maintaining the BM HSC pool. These evidences indicated that the heterogeneous CAR cells contain a variety of BM stromal progenitors and mature stromal cells, and serve as the main source of CXCL12 and SCF in BM.

❖ **$Grem1^+$ MSPCs**

Gremlin 1 (Grem1) can inhibit BMP signaling and is critical for new bone formation (Canalis et al., 2012; Khokha et al., 2003). Mouse BM $Grem1^+$ cells are fully negative for hematopoietic markers, but partially positive for CD105, CD140A, Sca1, and low $CD140A^+SCA1^+$ (Worthley et al., 2015). The $Grem1^+$ cells have a better self-renewal capacity than the $Nestin^+$ MSCs, which is confirmed by the higher frequency of CFU-Fs of the $Grem1^+$ cells (Worthley et al., 2015). However, the $Grem1^+$ cells can differentiate into osteoblasts and chondrocytes *in vitro*, but can not generate adipocytes (Worthley et al., 2015). Furthermore, both endogenous and transplanted BM $Grem1^+$ cells form new bones *in vivo* and contribute to the bone fracture healing (Worthley et al., 2015). Together, mouse BM $Grem1^+$ cells are considered as an osteo-restricted stem and progenitors and involve in new bone formation *in vivo*. The roles of BM $Grem1^+$ cells in the regulation of hematopoiesis are yet to be further investigated.

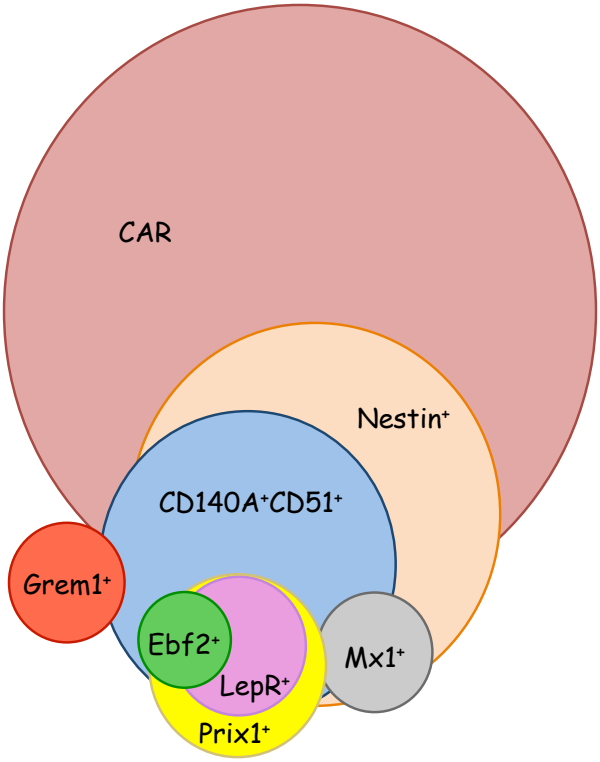

Figure 8. Relationships among the BM MSC/MSPC populations defined by using different genetic mouse models. CAR cells are highly express the chemokine CXCL12, and contain a variety of BM stromal progenitors and mature stromal cells. About 75% of $CD140A^+CD51^+$ MSCs are $Nestin^+$, whereas around 60% of $Nestin^+$ cells are $CD140A^+CD51^+$ (Pinho et al., 2013). About 70% $LepR^+$ MSCs express both CD140A and CD51 (Pinho et al., 2013). About 95% of $Prx1^+$ cells express CD140A (Ding and Morrison, 2013), and around half of the PαS cells ($CD140A^+Sca1^+$ MSCs) are positive for Prx1 expression (Greenbaum et al., 2013). About 44% of the $CD105^+CD140A^+$ stromal cells express Mx1 (Park et al., 2012). $Grem1^+$ MSPCs do not express Nestin but are partially positive for CD140A (Worthley et al., 2015). $Ebf2^+$ MSCs are fully positive for CD140A and CD51 (Qian et al., 2013).

1.1.2.3 Essential BM HSC niche soluble factors for maintaining normal hematopoiesis

❖ **CXCL12**

CXCL12 has been shown to maintain BM HSC pools through CXCL12-CXCR4 signal pathway (Sugiyama et al., 2006). The universal depletion of CXCL12 leads to a proliferation of BM hematopoietic progenitors along with a reduction of BM LT-HSCs in mice (Tzeng et al., 2011). However, CXCL12 secreted by different BM stromal cells exhibits different effects on hematopoiesis (Ding and Morrison, 2013; Greenbaum et al., 2013). For example, the depletion of CXCL12 in $Prx1^+$ MSCs leads to dramatic reductions of LT-HSCs and common lymphoid progenitors, along with a mobilization of HSPCs (Ding and Morrison, 2013; Greenbaum et al., 2013). The depletion of CXCL12 in $Tie2^+$ endothelial cells results in impaired BM HSCs with mild effect on other hematopoietic progenitors (Ding and Morrison, 2013; Greenbaum et al., 2013). The depletion of CXCL12 in $Osterix^+$ or $Col2.3^+$ osteoblasts leads to reduced lymphoid progenitors (Ding and Morrison, 2013; Greenbaum et al., 2013).

Moreover, a mobilization of HPCs is found when CXCL12 is specifically removed from Osterix$^+$ osteoblasts (Greenbaum et al., 2013). The conditional depletion of CXCL12 in NG2$^+$ pericytes induces the mobilization of quiescent BM HSCs into circulation and results in a reduction of BM HSCs (Asada et al., 2017). However, the depletion of CXCL12 in LepR$^+$ MSCs has little effect on the hematopoiesis in BM (Asada et al., 2017; Ding and Morrison, 2013). Additionally, no effect on BM hematopoiesis is found when CXCL12 is depleted in osteocalcin (Oc)$^+$ mature osteoblasts or Nestin$^+$ cells or Vav1$^+$ hematopoietic cells (Ding and Morrison, 2013; Greenbaum et al., 2013). These evidences suggest that CXCL12 produced by various BM cells differentially regulates BM hematopoiesis and that CXCL12 is a crucial BM niche factor (**Table 2**).

Table 2. Effects of CXCL12 on BM hematopoiesis

CXCL12 depletion in specific cells	**Effects on BM hematopoiesis**	**References**
Cxcl12 knock-out	LT-HSCs ↓, and ST-HSCs ↑	Tzeng et al., 2011
Prx1-Cre	LT-HSCs ↓, CLPs ↓ Mobilizations of HSCs into circulation ↑	Ding and Morrison, 2013; Greenbaum et al., 2013
Tie2-Cre	HSCs ↓	Ding and Morrison, 2013; Greenbaum et al., 2013
Osx-Cre	Pre-pro B cells ↓, CLP ↓ Mobilizations of HSCs into circulation ↑	Greenbaum et al., 2013
Col2.3-Cre	Lymphoid progenitors ↓	Ding and Morrison, 2013
NG2-Cre	HSCs ↓ Mobilizations of HSCs into circulation ↑	Asada et al., 2017
Lepr-Cre	No obvious effect	Ding and Morrison, 2013; Asada et al., 2017
Oc-Cre	No obvious effect	Greenbaum et al., 2013
Nestin-Cre	No obvious effect	Ding and Morrison, 2013
Vav1-Cre	No obvious effect	Ding and Morrison, 2013

❖ SCF

SCF is also known as Kit ligand (Kitl) or Steel (Sl) factor, which binds c-Kit expressing HSPCs (Williams et al., 1990; Zsebo et al., 1990). Mice lacking membrane-bound SCF display impaired hematopoiesis, which can be restored after the transplantation of their BM cells into a normal BM niche (Barker, 1994, 1997). These studies indicate critical roles of membrane-bound SCF in the BM niche for maintaining the HSC pool. Nevertheless, the SCF secreted by different BM stromal cells differentially regulates HSCs. The conditional depletion of SCF in Tie2$^+$ endothelial cells (Ding et al., 2012) or LepR$^+$ MSCs (Asada et al., 2017; Ding et al., 2012) or NG2$^+$ pericytes (Asada et al., 2017) leads to a dramatic reduction of BM HSCs. However, the SCF secreted by Nestin$^+$ cells, Col2.3$^+$ osteoblasts, or Vav1$^+$ hematopoietic cells is dispensable for BM hematopoiesis (Ding et al., 2012). Therefore, SCF secreted from vascular niche is essential for maintaining BM HSCs (**Table 3**).

Table 3. Effects of SCF on BM hematopoiesis

SCF depletion in specific cells	Effects on BM hematopoiesis	References
Tie2-Cre	Normal BM cellularity; LT-HSCs ↓	Ding et al., 2012
Lepr-Cre	Normal BM cellularity; LT-HSCs ↓	Ding et al., 2012; Asada et al., 2017
NG2-Cre	Normal BM cellularity; LT-HSCs ↓	Asada et al., 2017
Nestin-Cre	No obvious effect	Ding et al., 2012
Col2.3-Cre	No obvious effect	Ding et al., 2012
Vav1-Cre	No obvious effect	Ding et al., 2012

❖ **THPO**

THPO is mainly secreted by liver, kidney, and BM stromal cells (Ishikawa et al., 1998; McIntosh and Kaushansky, 2008; Sungaran et al., 1997). THPO binds to its receptor MPL which is expressed in LT-HSCs to inactivate the cell cycling of HSCs (Kimura et al., 1998; Qian et al., 2007; Yoshihara et al., 2007). Furthermore, the BM in-situ secretion of THPO is detected in osteoblasts at endosteal area, strengthening BM LT-HSC quiescence through the in-situ THPO/MPL signaling (Yoshihara et al., 2007). These evidences suggest that THPO is a critical BM niche factor to maintain HSC quiescence in adult.

1.1.3 Other BM microenvironmental cells

Sympathetic nerves have been shown to play a role in maintaining and restoring normal hematopoiesis by regulating soluble factors like CXCL12 (Lucas et al., 2013; Mendez-Ferrer et al., 2008). Schwann cells in BM contribute to the maintenance of HSC quiescence through the activating TGF-β/Smad signaling in HSCs (Yamazaki et al., 2011).

In summary, HSCs reside in BM niche and are tightly regulated by direct cell-interaction with BM niche cells or soluble factors secreted by the niche cells. Knowledge of cellular and molecular mechanisms underlying the BM niche regulation in normal hematopoiesis is fundamental for exploring the roles of different BM niche components in hematological malignancies.

1.2 BM niche in myeloid malignancies

In 1889, Dr. Paget formulated the famous “seed and soil” hypothesis to explain why metastases of breast cancer can spread to specific organs (Paget, 1989). In addition to cancer metastasis, the “seed and soil” hypothesis also suggests a dual cause of cancers, which can be induced either from the cancer cells (seed) and/or their environment (soil). Here, I summarize the recent evidence on BM niche contributions to myeloid malignancies, such as myeloproliferative neoplasms (MPN), acute myeloid leukemia (AML), and myelodysplastic syndrome (MDS), and describe the crosstalk between the leukemic cells (seed) and the BM niche (soil).

1.2.1 BM niche contribution to MPN

MPN are heterogeneous diseases characterized by at least one myeloid lineage with chronic clonal proliferation in BM. According to the 2016 World Health Organization (WHO) classification, MPN include chronic myeloid leukemia (CML) with BCR-ABL expression, and BCR-ABL$^-$ MPN, such as chronic neutrophilic leukemia (CNL), polycythemia vera (PV), primary myelofibrosis (PMF), essential thrombocythemia (ET), chronic eosinophilic leukemia, and unclassified MPN (Arber et al., 2016).

1.2.1.1 Intrinsic regulation of CML

CML is a clonal disease characterized by the appearance of the Philadelphia chromosome which is caused by the translocation of t(9;22)(q34;q11) (Groffen et al., 1984). This translocation results in the expression of the *BCR-ABL* oncogene, a constantly active tyrosine kinase (Groffen et al., 1984). Treatments with tyrosine kinase inhibitors (TKIs), such as imatinib, greatly improve the survivals of CML patients (Hochhaus et al., 2017). However, many patients do not respond to imatinib therapy, and some patients have to stop imatinib because of serious side-effects (Gambacorti-Passerini et al., 2011). Despite the increased use of new TKIs, such as dasatinib, nilotinib, radotinib, and bosutinib, many patients are still facing drug resistance, serious side-effects, and disease relapse, particularly after TKI discontinuation (Ongoren et al., 2017). Therefore, further understanding the underlying mechanisms of CML and searching for alternative drugs are in great need to improve patient outcomes.

CML has been identified as a hematopoietic stem cell disease by using the BCR-ABL CML mouse model, (Schemionek et al., 2010). The mouse CML can be induced by BCR-ABL$^+$ LSK, but not by BCR-ABL$^+$ LSK$^-$ progenitors or BCR-ABL$^+$ mature granulocytes (Schemionek et al., 2010). Many signaling pathways including β-catenin, c-MYC and declined p53 are involved in the development of CML (Abraham et al., 2016; Eiring et al., 2015). *Alox5*, coding for arachidonate 5-lipoxygenase (5-LO), has been demonstrated to be critical for leukemic stem cell survival in the CML mice (Chen et al., 2009). The deficiency of *Alox5* or the application of a 5-LO inhibitor impairs the LSCs and prolongs the CML mouse survival (Chen et al., 2009). However, our recent study using CML patient samples suggests that the leukotriene inhibitors acting on 5-LO and cysteinyl leukotriene receptor-1 play minor role in human LSC survival in vitro (Dolinska et al., 2017). More studies with a bigger patient cohort with CML are necessary to further address the therapeutic effects of leukotriene inhibition on CML LSC persistence.

1.2.1.2 Remodeling effects of leukemic cells on the BM niche

The involvement of the BM niche in leukemia development has drawn much attention to characterization of leukemic BM niche and search for alternative treatments targeting BM niche. Two classic MPN mouse models are used to study the roles of the BM niche in the regulation of MPN. One is the CML mouse model induced by BCR-ABL expression in hematopoietic cells. The other mouse model is induced by the mutated gene *janus kinase 2*

(*JAK2*(V617F)), which is detected in most of BCR-ABL negative MPN patients (Baxter et al., 2005; Kralovics et al., 2005).

1.2.1.2.1 BM niche remodeling in CML

Mouse CML cells often induce an overproduction of BM osteoblast progenitor cells and promote myelofibrosis in the CML BM. The increase of osteoblasts is mediated by cell-cell attachments and an increase of the soluble factors including chemokine ligand 3 (CCL3) and THPO in the CML BM niche (Schepers et al., 2013). These CML osteoblast progenitor cells exhibit activated TGF-β, Notch, Wnt, and inflammatory signaling, which is involved in the process of myelofibrosis (Schepers et al., 2013). The gene expression of *Cxcl12*, *Scf*, and *Angpt1* is reduced in the osteoblast progenitors of the CML mice, contributing to a decline of normal hematopoietic supportive function *in vivo* (Schepers et al., 2013). Together, these results indicate that the mouse CML cells gradually educate their BM niche into a leukemic niche to favor the LSC expansions (Schepers et al., 2013) (**Figure 9**).

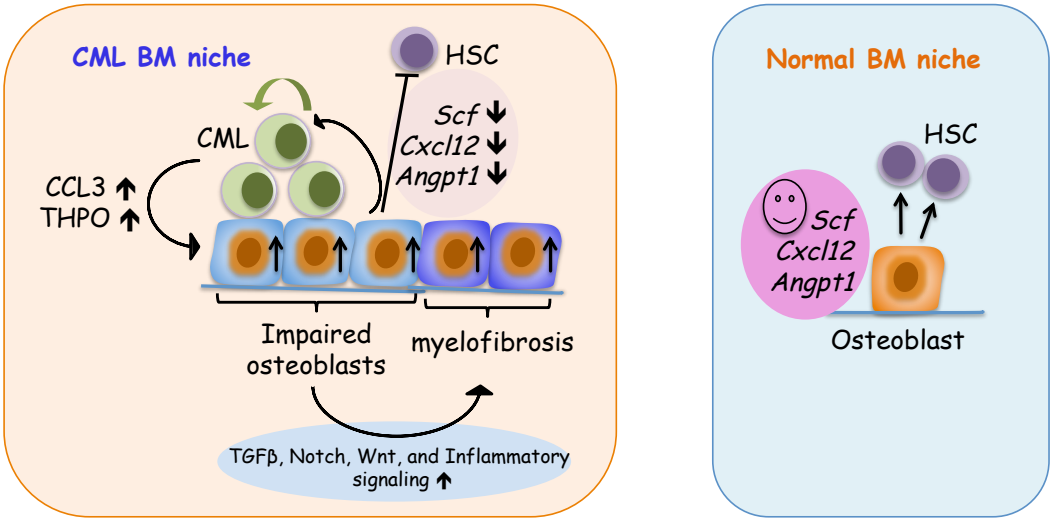

Figure 9. BM niche remodeling in CML. Mouse CML cells gradually educate their BM niche into a leukemic niche to favor the LSC expansions.

1.2.1.2.2 BM niche alterations in *JAK2*(V617F) MPN

BM niche contribution to MPN progression has also shown in the *JAK2* mutated mouse model. Sympathetic signaling and BM Nestin$^+$ MSPCs are reduced in MPN patients and *JAK2*(V617F) mutated MPN mice (Arranz et al., 2014). In the MPN mice, *JAK2*(V617F) mutated malignant hematopoietic cells induce the neuropathy and a decline of BM Nestin$^+$ MSPCs in the BM *via* IL-1β production and impaired adrenergic signaling (Arranz et al., 2014). Application of β3-adrenergic agonist or neuroprotective medicine can protect BM Nestin$^+$ MSPCs from apoptosis, followed by a prevention of the MPN (Arranz et al., 2014). These data provide evidence for the niche disruption induced by the *JAK2*(V617F) mutated malignant cells, which may in turn facilitate the MPN progression (**Figure 10A**).

The *JAK2*(V617F) mutation has been reported to be detected in the endothelial cells of MPN patients, indicating the potential involvement of endothelial cells in MPN development (Rosti et al., 2013; Teofili et al., 2011). Using the (*Tie2-Cre; JAK2*(V617F))

mouse model, the *JAK2*(V617F) mutation can be specifically activated in the endothelial cells. The mutated endothelial cells support *JAK2*(V617F) HSCs better than wild type endothelial cells (Zhan et al., 2017). Compared to wild type endothelial cells, the *JAK2*(V617F) mutated endothelial cells exhibit increased CXCL12, SCF, and THPO/MPL signaling to promote the expansion of *JAK2*(V617F) HSCs *in vivo* (Zhan et al., 2017). These results indicate that the *JAK2*(V617F) mutated endothelial cells promote the growth of the *JAK2*(V617F) mutated HSCs and may contribute to the MPN progression (**Figure 10B**).

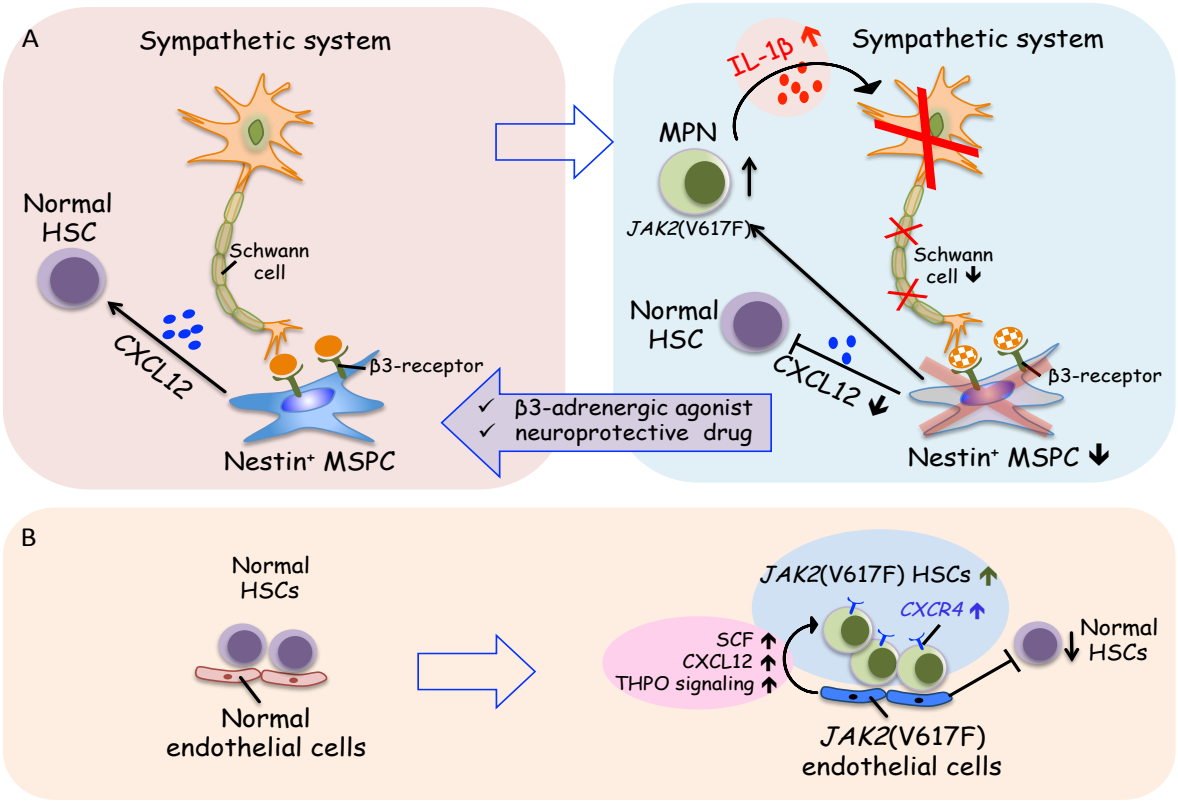

Figure 10. Roles of the BM niche in the *JAK2*(V617F) mutation-induced MPN. (A) The sympathetic nervous system is damaged by the *JAK2*(V617F) mutated HSCs, which facilitates the MPN progression. **(B)** The *JAK2*(V617F) mutated endothelial cells promote the growth of the *JAK2*(V617F) mutated HSCs and accelerate to the MPN progression.

1.2.1.3 Instructive roles of BM niche in the pathogenesis of MPN

1.2.1.3.1 Gene deficiencies in BM niche can induce MPN-like disease

There are increasing evidence showing critical roles of the BM niche in the MPN pathogenesis (**Figure 11**). Mice with Retinoic acid receptor γ (RARγ) deficiency present the MPN-like phenotype, including increased myeloid progenitors and mature myeloid cells, BM hypercellularity, and myeloid cell infiltration in other tissues (Walkley et al., 2007a). However, transplantation experiments suggest that the phenotype of the mice is not caused by the loss of RARγ in the hematopoietic cells, but in the BM niche cells (Walkley et al., 2007a). TNF signaling is increased in the RARγ deficient BM niche, and mediates the pathogenesis of the disease (**Figure 11A**) (Walkley et al., 2007a). Moreover, the RARγ deficient BM niche is absolutely required to maintain the MPN phenotype in secondary recipients, indicating critical roles of BM niche in the initiation of the MPN (Walkley et al., 2007a). In addition,

retinoblastoma protein (RB) deficiency in mouse BM cells leads to increased myelopoiesis in *Mx-cre;pRb*$^{fl/fl}$ mice (Walkley et al., 2007b). The Rb deficient mice exhibit increased myeloid cells, cKit^{+} HSPCs, and total mononuclear cells in the BM (Walkley et al., 2007b). Nevertheless, the depletion of RB in the myelo-restricted hematopoietic cells or in the BM niche alone cannot induce the phenotype (Walkley et al., 2007b). This study indicates the importance of the BM niche homeostasis in maintaining normal hematopoiesis and MPN pathogenesis.

1.2.1.3.2 *Ptpn11*^{E76K} activating mutation in BM MSPCs induces MPN

PTPN11 mutation is often detected in patients with MPN. The patients with *PTPN11* activating mutation are prone to develop leukemia, especially juvenile myelomonocytic leukaemia (JMML), a childhood MPN (Roberts et al., 2013). The activation of *Ptpn11*^{E76K} in Prx1^{+} MSCs, or Lepr^{+} MSCs, or Nestin^{+} MSCs, or Osx1^{+} osteoprogenitors alone induces the MPN (Dong et al., 2016). However, Ocn^{+} mature osteoblasts or VE-Cadherin^{+} endothelial cells with the activated *Ptpn11*^{E76K} mutation fail to induce the MPN (Dong et al., 2016), suggesting the critical role of Ptpn11 signaling in mesenchymal progenitor cells for the MPN initiation. The *Ptpn11*^{E76K} mutated MPN cells produce IL-1β, whereas the *Ptpn11*^{E76K} mutated BM MSPCs produce a higher level of CCL3, which may promote the MPN progression, since CCL3 antagonists can restore the MPN phenotype in these mice (Dong et al., 2016). This study provides novel evidence for the critical role of BM niche in the initiation and progression of the MPN (**Figure 11B**).

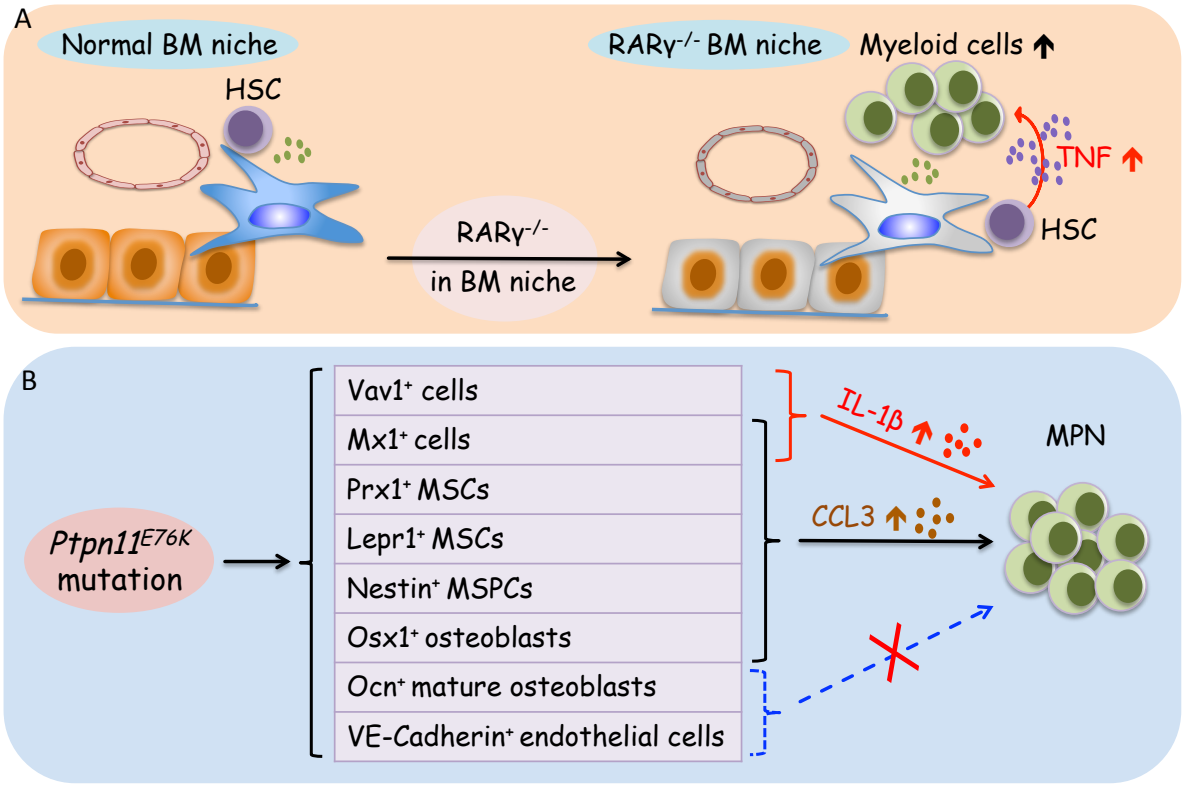

Figure 11. Instructive roles of the BM niche to MPN pathogenesis. (A) The loss of RARγ in the BM niche drives the MPN in mice. **(B)** The *Ptpn11*^{E76K} mutation in mouse BM hematopoietic cells or MSPCs leads to the MPN development through the productions of IL-1β and CCL3, respectively.

1.2.1.3.3 SIPA1 deficiency in MPN development

Signal-induced proliferation-associated gene-1 (SIPA1) is a specific Rap1 GTPase-activating protein (Rap1GAP), which inactivates Rap1 signaling to regulate cell proliferation, adhesion, and survival (**Figure 12**) (Jin et al., 2006; Kometani et al., 2004; Kurachi et al., 1997; Minato and Hattori, 2009). SIPA1 expression is detected in lymphocytes and LSKs (Hattori et al., 1995; Ishida et al., 2003). Abnormal expressions of SIPA1 are related to the pathogenesis of several solid cancers, such as cervical cancer (Brooks et al., 2010), breast cancer (Ugenskiene et al., 2016; Yi and Li, 2014; Zhang et al., 2015), and prostate cancer (Park et al., 2005). *SIPA1* point mutations are detected in JMML patients with unclear pathological functions (Yoshida et al., 2008), and in AML patients with the CEBPA mutation (Dolnik et al., 2012).

The *in vivo* function of SIPA1 in hematopoiesis has been demonstrated in a *Sipa1* gene deleted mice (*Sipa1*$^{-/-}$) (Ishida et al., 2003). *Sipa1*$^{-/-}$ mice have normal hematopoiesis and develop normally until five months of age. However, most of the *Sipa1*$^{-/-}$ mice develop age-dependent myeloid disorders with very heterogeneous phenotypes, such as CML-like MPN, MDS-like, and undefined hematological disorders with blast cells of diverse lineages (Ishida et al., 2003). Endogenous SIPA1 is reduced in the HPCs with an overexpression of BCR-ABL, followed by enhanced Rap1 signaling in the cells (Kometani et al., 2006). Interestingly, *Sipa1* is not only expressed by hematopoietic cells, but also by different BM stromal cells, such as MSCs, MPCs, endothelial cells, and mature stromal cells (indicated in attached Paper **I**), open the possibility of the effect of SIPA1 expression in BM niche on the development of the MPN.

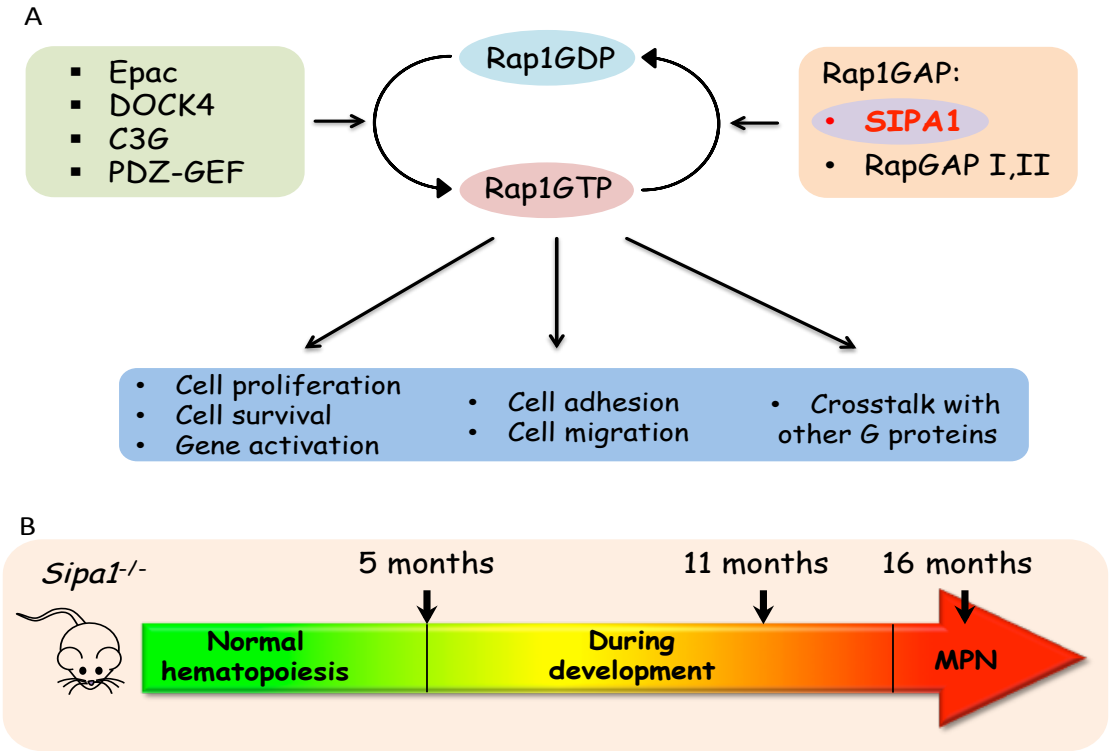

Figure 12. SIPA1 signaling and the *Sipa1*$^{-/-}$ mouse model. (A) SIPA1 is a specific Rap1GAP, which inactivates Rap1 signaling to regulate cell proliferation, adhesion, and survival. **(B)** *Sipa1*$^{-/-}$ mice have normal hematopoiesis and develop normally until five months of age. Most of the *Sipa1*$^{-/-}$ mice develop myeloid disorders with very heterogeneous phenotypes at 16 months of age.

1.2.2 BM niche involvement in MDS and AML development

1.2.2.1 AML-induced alterations of BM niche

It has been shown that the MLL-AF9$^+$ AML mice exhibit increased numbers of endothelial cells, MSPCs (Lin$^-$CD31$^-$CD140A$^+$CD51$^+$), Nestin$^+$ MSPCs, and LepR$^+$ MSCs, but reduced NG2$^+$ pericytes in the BM (Hanoun et al., 2014). In a xenograft NSG mouse model, human AML cells are engrafted in a normal human bone biopsy implanted in the NSG mouse and shift the normal niche into an osteogenic niche (Battula et al., 2017). In addition, AML cells induce the lipolysis of adipocytes to generate fatty acids which can be consumed by the AML cells for their survival (Shafat et al., 2017). The BM stromal cells of newly diagnosed AML patients present reduced gene expressions of *ANGPT1*, *KITL*, and *THPO*, indicating an impaired hematopoiesis supportive function of the AML stromal cells (Desbourdes et al., 2017). Together, these studies indicate that AML cells alter the compositions and functions of the BM niche to form a leukemic niche favoring their own growth.

1.2.2.2 Dysfunctional BM HSC niche induces leukemic transformation

The deficient BM niche can also alter normal hematopoiesis and induce leukemia (**Figure 13**). The conditional depletion of *Dicer1* in the osterix$^+$ osteoprogenitors leads to MDS in the mice and some of the MDS mice develop secondary AML (Raaijmakers et al., 2010). A reduced expression of the *Shwachman-Diamond-Bodian syndrome* (*Sbds*) gene is detected in the osteoprogenitors after the depletion of *Dicer1*, which is related to the MDS and AML in the mice (Raaijmakers et al., 2010). The mice deficient in *Sbds* in osteoprogenitors develop MDS formation, which is mediated by the activated p53-S100A8/9-TLR4 inflammatory signaling in BM (Zambetti et al., 2016). Similarly, an enhanced inflammatory signaling has been detected in human BM stromal cells with low risk MDS, indicating the involvement of inflammatory signaling in leukemia development (Chen et al., 2016).

It has been shown that the intrinsic Wnt/β-catenin signaling in the AML cells is critical for the expansion of leukemic stem cells (Wang et al., 2010). However, the abnormal Wnt/β-catenin signaling in the osteoblasts also contributes to AML progression. Using the *Ctnnb1*^{CAosb} mouse model, the constitutive activation of a β-catenin mutant in the osteoblasts leads to AML. This leukemic transformation is promoted by the activated Notch signaling in HSCs, which is attributed to the increased expression of the Notch ligand jagged1 in the osteoblasts along with the increased Notch receptors in the HSPCs (Kode et al., 2014). Moreover, FoxO1 expression in the osteoblasts enhances the activated β-catenin signaling with an up-regulated jagged1 expression in the osteoblasts and instigates the AML progression (Kode et al., 2016; Sykes et al., 2011). In turn, by using the *Apc*$^{del/+}$ mouse model, it has been shown that the reduced β-catenin in the mouse BM niche prevents the MDS progression (Stoddart et al., 2017). Collectively, these studies suggested that the genetically impaired BM niche can induce MDS and/or AML in mice.

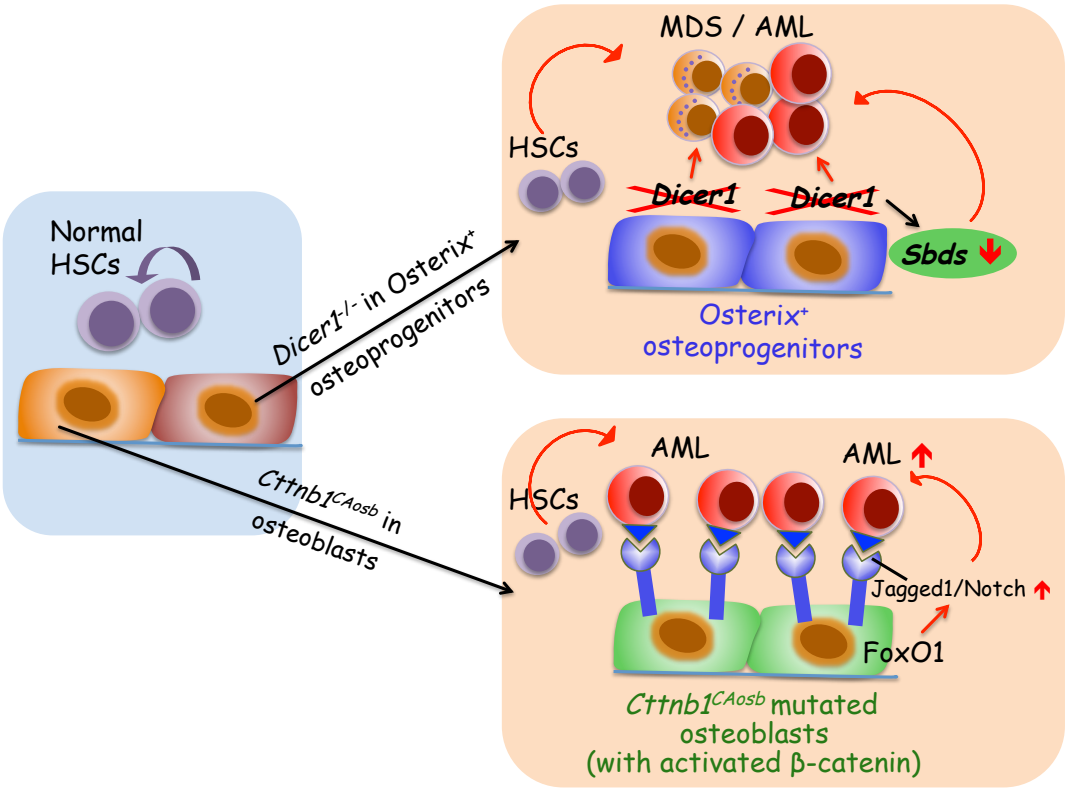

Figure 13 Impaired BM HSC niche induce MDS and/or AML. The conditional depletion of *Dicer1* in the osterix^{+} osteoprogenitors leads to MDS in the mice and some of the MDS mice develop secondary AML (upper panel). The constitutive activation of a β-catenin mutant in the osteoblasts leads to the AML in the *Ctnnb1*^{CAosb} mice (lower panel).

1.2.2.3 BM niche protection to AML cell survival during chemotherapy

BM stromal cells express CXCL12, which can bind to its receptor CXCR4 that is expressed in hematopoietic cells to regulate HSC activities, such as homing and maintaining HSC quiescence (Sugiyama et al., 2006). Endogenous CXCR4 is also detected in AML cells, and the application of CXCL12 cytokine in culture promotes the survival of the AML cells (Tavor et al., 2004). A CXCR4 antagonist has previously been developed to hinder human immunodeficiency virus-1 (HIV-1) activities, and is now applied for leukemia treatments through inhibition of CXCL12/CXCR4 signaling (Oberlin et al., 1996). The application of the CXCR4 antagonist leads to impaired homing, engraftment, and infiltration of AML cells in mice, inhibits AML cell growth and prolongs mouse survival (Nervi et al., 2009; Tavor et al., 2004; Zeng et al., 2009). It has been shown that the BM-derived stromal cell line HS-5 protects AML cells from chemotherapy-induced apoptosis under co-culture condition (Garrido et al., 2001). This protection is mediated by the CXCL12/CXCR4 signaling and the activated mammalian target of rapamycin (mTOR) signaling between the stromal cells and AML cells (Braun et al., 2016). In addition, AML cells bind to BM stromal cells through the very late antigen-4 (VLA-4) expressed in leukemic cells and fibronectin receptors in stromal cells to escape from chemotherapy-induced apoptosis (Bendall et al., 1993; Bendall et al., 1998; Matsunaga et al., 2003). The combination treatment with anti-VLA-4 antibody and cytosine arabinoside accelerates the apoptosis of the AML cells and diminishes the minimal

residual disease of AML (Matsunaga et al., 2003). Together, these studies indicate that the protective roles of BM stromal cells for AML cells during chemotherapy, and that targeting the interactions may be promising for the treatment of leukemia.

1.2.3 Distinct regulations of leukemic cells by BM niche in different types of leukemia

Previous studies have indicated that the distinct roles of the BM niche in leukemia progression in different types of leukemia (Krause et al., 2013). The continuous activation of PTH signaling in BM osteoblasts leads to a reduction of BCR-ABL$^+$ CML cells, but promotes the MLL-AF9$^+$ AML cell expansion in the mice (Krause et al., 2013). The opposite outcomes in the CML and AML mice indicate the distinct role of the disease-specialized BM niche in leukemia progressions (Krause et al., 2013).

1.3 Significance of studies on BM HSC niche

HSCs reside in the BM niche to self-renew and generate mature blood cells in postnatal life. The BM niche regulates HSC activities through secreting cytokines, growth factors, and matrix proteins, as well as cell-cell interactions. Studies have shown that LSCs can remodel the normal BM niche into a leukemic niche, which in turn the abnormal BM niche can also induce leukemia and promote leukemia progression in mice. Evidence has shown that interfering with these interactions can restore or attenuate disease development in mice. Better understanding of the interactions between LSCs and their BM niche may help to design alternative therapies targeting BM niche for the treatment of leukemia.

2 Aim of the Thesis

The overall aim of the thesis is to understand the roles of BM mesenchymal stem and progenitor cells in the regulation of myeloid malignancies, including MPN and AML.

- **Study I**

To determine whether BM microenvironment contributes to the pathogenesis of MPN by using a *Sipa1* deficient mouse model.

- **Study II**

To identify whether and how BM stromal cells contribute to AML niche formation and the dynamic role of the BM niche cells in AML development by using a transplantation-induced MLL-AF9 AML mouse model.

- **Study III**

To study the effect of different cultivation methods on the immunophenotype, gene expression, and proliferation of mouse MSCs.

- **Study IV**

To examine the expressions of leukotriene signaling molecules of CML leukemic stem and progenitor cells by single cell assays and the effects of leukotriene inhibitors on the survival of CML LSCs using pharmaceutical inhibitors targeting ALOX5 or cysteinyl leukotriene receptor 1 *in vitro*.

3 Methodological Approaches

All animal experiments included in this thesis have been approved by Karolinska Institutet ethic committee with the ethical number of S40-14, and patient sample collections have been approved with permission of 2012/4:10, 2013/3:1, and 2013/1248-31/4 at Stockholm. Here I describe the main methods applied to the studies.

3.1 Mouse MSC FACS isolation and analysis

MSCs isolation technique has been evolved for several decades from previously plastic culture selection to nowadays prospective isolation of primary MSCs. Plastic culture selected MSCs are obtained by seeding bulk BM cells into culture and washing away non-adherent cells after several passages (Prockop, 1997). This procedure is easy to perform to get abundant MSCs, however, it presents several disadvantages as our increased knowledge about MSCs. First, hematopoietic cells can not be completely removed in plastic culture even after several passages, leads to a residual hematopoietic cells mixed with the culture-selected MSCs. Second, plastic culture can alter the phenotype of primary MSCs, which is indicated by the fact that primary human and mouse MSCs do not express CD44, but acquire CD44 expression after culture (Qian et al., 2012). Third, culture selected cells are very heterogeneous cell population, and not all culture adherent stromal cells possess the MSC characteristics, including self-renewal, expressing MSC marker, and multi-lineage differentiation capacities (Horwitz and Keating, 2000). Fourth, most of MSCs are located at endosteum area and can not be easily obtained through simply crushing the bones, but through treating the bone with collagenase II and trypsin-EDTA. Therefore, in paper **I**, **II**, and **III**, we performed the mouse BM MSC isolation following our standard isolation protocol tested by Qian (Qian et al., 2012) to obtain freshly sorted MSCs for our studies.

To make sure with the qualities of freshly isolated MSC, several details need to be pay attention to. First, high serum (10% to 20%) in the isolation media is required to keep good viabilities of the sorted MSCs. Second, low temperature (on ice or 4°C) can also keep MSCs with a good viability. Third, mixing well of bone fragments during incubation with collagenase and trypsin-EDTA is important, which helps with the MSC dissociation from bones. Fourth, sheep anti-rat IgG Dynalbeads®, which has been designed to isolate cell populations through binding to rat-anti-mouse antibodies, needs to be prewashed before hand to remove the possible ingredient which might be harmful to MSCs. Finally, fluorescence-activated cell sorting (FACS) need to be performed as soon as possible to preserve functional MSCs. FACS is a laser based cell sorter that can separate heterogeneous cells one by one into 1 to 4 enriched cell populations according to the activated fluorescence on the cells (Julius et al., 1972). After staining the cells with specific fluorescence conjugated antibodies, samples are acquired by sorter FACSAriaIII (BD), and data are presented in FACSDiva (BD) software, where each dot represented one cell from the sample. The cell sorting is gated based on fluorescent minus one (FMO) and the sorted cells are freshly collected for colony assay or gene expression assay.

3.2 Human MSC FACS isolation

Concerning about the disadvantages of plastic culture selected MSCs, we also perform freshly isolation of patient BM MSC following our standard isolation protocol (Qian et al., 2012). To collect BM mononuclear cells from patient samples, we apply Lymphoprep (Axis Sield) to samples with density centrifugation. Nevertheless, given the fact the density of BM stromal cells are heavier than that of hematopoietic mononuclear cells, we collect both the interface mononuclear cells, and those in the liquid between the interface mononuclear layer and red blood cell sediments. In addition, we use negative markers for sorting human MSCs, including non-hematopoietic ($CD45^-CD235^-$), non-endothelial ($CD31^-$), and $CD44^-$, due to the fact that primary MSCs are enriched in $CD44^-$ fraction (Qian et al., 2012). However, we also include MSC positive markers, such as CD146 and CD271, to confirm our $CD44^-$ MSCs.

3.3 CFU-F assay

BM MSCs have the capacity to form fibroblast-like colony (> 50 cells) in culture, whereas mature stromal cells can not survive in culture and some stromal progenitor cells can only form clusters (< 50 cells). Therefore, CFU-F assay is a method for assessing functional MSCs *in vitro* (Friedenstein et al., 1970). In the attached papers, we performed CFU-F assay following our standard protocol (Qian et al., 2012) to address two questions. First, to evaluate whether the functionally defined MSC were altered, we seeded unfractionated mouse BM cells containing those collected from bone fragment dissociation for CFU-F assay. Second, to identify CFU-F capacity of pure MSCs from normal and leukemic mice, we seeded freshly sorted mouse BM MSCs ($Lin^-CD45^-CD44^-CD51^+SCA1^+$) for CFU-F assay.

3.4 *In vitro* co-culture and CFU-C assay

In vitro co-culture hematopoietic stem and progenitor cells (HSPCs) and BM stromal cells can be applied to evaluate the hematopoietic supportive function of BM stromal cells (Nakamura et al., 2010). In paper **I**, to avoid the effects of long time co-culture induced declines of CFU-C capacity of HSPCs, we co-cultured the freshly sorted LSKs with freshly sorted BM stromal cells for only 2.5 days. After co-culture, the effects from BM stromal cells on hematopoietic stem and progenitor cells were accessed by CFU-C assay. CFU-C assay is a classic colony assay based on the morphology of the formed colony to evaluate the differentiation capacity of HSPCs in semi-liquid media containing specific cytokines (Bradley and Metcalf, 1966). In this semi-liquid media, HSPCs are seeded at a low density to reach well separations from each other to form single cell colony. The unipotent HPCs are identified by the morphology of the colony containing single lineages, such as granulocytes (CFU-G), monocytes (CFU-M), and erythrocytes (CFU-E). The bipotent HPCs and multi-potent HSPCs are identified by the morphology of CFU-GM and CFU-GME, respectively. However, the CFU-C assay also have its limitations. First, distinguishing the morphology of different colony requires substansive experience, we should score the colonies before they over-confluence and take counting error into consideration. Second, culture conditions, such as temperature and humidity, can also affect colony formations. Third, abnormal HSPCs

defected in responding to cytokine stimulation can not be evaluated by CFU-C assay (Metcalf, 1993).

3.5 *In vitro* multi-lineage differentiation assay

MSCs have capacities to differentiate into osteoblasts, adipocytes, and chondrocytes (Dominici et al., 2006). However, during pathogenesis of myeloid disease, the multi-lineage differentiation capacity can change (Battula et al., 2017; Hanoun et al., 2014). In attached paper **I** and **II**, we performed multi-lineage differentiation assays following our standard protocol (Qian et al., 2013) to evaluate the functional alterations of MSCs from the MPN or AML mice compared with that of normal mice. Mouse MSCs were freshly sorted and cultured in mouse complete Mesencult media (R&D or Stem Cell Techn.) for 7-10 days. The cells were then collected and cultured in differentiation induction media for osteoblast, adipocyte, and chondrocyte differentiations.

The evaluation of osteoblast differentiation is based on newly formed calcium deposition, which bind to Alizarin Red S to form Alizarin Red S-calcium compound with a red color. This mineralization complex is further dissolved by acetic acid and quantified using plate reader at absorbance at 405 nm. Adipocyte differentiation is evaluated by the fat lipid formation in the cells. The fat lipid is recognized by its special morphology, however, the fat lipid can be easily dissolved during the process of histology staining such as hematoxylin and eosin (HE) staining. Therefore, we take advantage of Oil Red O, a fat soluble dye, which exhibits enhanced solubility in fat lipid than in the dye solvents, and stains the fat lipid with a red color. Unlike the osteoblast and adipocyte differentiations performed under normoxic condition, chondrocyte differentiation is performed under hypoxic condition (1% O_2). Chondrocyte differentiation can be induced in monolayer culture, but can reach a better induction in pellet culture (3D culture) (Zhang et al., 2010). The chondrocyte differentiation is confirmed by Toluidine blue, which stains the specific matrix proteins in the differentiated chondrocyte cytoplasm with a purple color.

3.6 Transplantation experiments

BM transplantation is a procedure that replacing endogenous hematopoietic cells with newly donor cells. Irradiation is normally applied to the recipient mice to empty BM space for the transplantation of normal hematopoietic donor cells. Lethally irradiation can maximally clear up endogenous hematopoietic cells to reach a high level of donor engraftment, whereas sublethally irradiation can obtain a lower engraftment of donor cells with less harm for endogenous cells. However, transplantation of immortalized leukemic cells may not need to irradiate the recipient mice, since leukemic cells can compete with normal hematopoietic cells and occupy the space in BM with time.

In paper **I**, we designed transplantation experiments to determine whether the development of the MPN was attributed to *Sipa1* deficiency in the hematopoietic cells or the BM niche. Two doses of irradiation were applied before the transplantations to test our hypothesis. First, in order to maintain the physiological function of the recipient BM niche,

and meanwhile obtain reasonable donor cell engraftment, we performed sublethally irradiation (6 Gy) before transplantation. Second, to examine the donor hematopoietic activity in a clean BM niche without affection from endogenous hematopoietic cells, we applied lethally irradiation (9.5 Gy) to the recipient mice before transplantation. The $CD45.1^+$ or $CD45.2^+$ BM cells were enriched by MACS using CD45 microbeads. These transplantation experiments provided the possibility to determine whether normal hematopoietic cells could develop MPN in *Sipa1*$^{-/-}$ BM niche. On the other hand, the *Sipa1*$^{-/-}$ cells were transplanted into lethally irradiated CD45.1 *Sipa1*$^{+/+}$ mice to test whether *Sipa1*$^{-/-}$ hematopoietic cells could develop MPN in a normal BM niche.

In paper **II**, to study BM niche contributions on AML progression, we set up the AML mouse model by intravenous transplantation of the MLL-AF9 retrovirus transduced mouse BM KIT^+ cells after clonally propagation in complete methylcellulose M3434 as reported (Somervaille and Cleary, 2006). No irradiation was performed to set up this AML mouse model. To study BM niche alterations, we transplanted MLL-$AF9^+$ AML cells to wild type CD45.2 C57BL6J mice. To determine the involvement of $Ebf2^+$ MSCs and their derived progenies in the AML development, MLL-$AF9^+$ AML cells were transplanted into *Ebf2-Egfp* reporter mice and triple transgenic Tg (*Ebf2-Egfp* x *Ebf2-Cre*^{ER} x *Rosa26-*^{loxp}*Stop*^{loxp}*-Tomato*) mice, respectively. To further evaluate the critical roles of $Ebf2^+$ MSCs in the AML development, MLL-$AF9^+$ AML cells were transplanted into Tg (*Ebf2-Cre*^{ER} x *Rosa26-*^{loxp}*Stop*^{loxp}*-Dta*) mice. Primary BM or PB mononuclear cells from AML patients were transplanted to sublethally irradiated (2.2 Gy) NSG mice through intra femoral injection to set up AML in immunosuppressive NSG mice.

3.7 Analysis of hematopoietic cells after transplantation

Analysis of blood reconstitution after transplantation is an essential method to check transplantation efficiency. In attached study **I** and **II**, to monitor hematopoietic changes, peripheral blood (PB) was collected from the tail vain in EDTA-coated tubes after transplantation, and was counted in an automated cell counter for blood components. The blood cell morphology was analyzed by blood smears and cytospins, which were stained by May-Grünwald-Giemsa. Flow cytometry analysis was performed to analyze hematopoietic lineage components in BM and blood based on specific antigen expression on different hematopoietic cells. Mature hematopoietic lineage cells include myeloid cells ($CD11B^+GR1^+$ and $CD11B^+GR1^-$ monocytes), B cells ($CD19^+$), T cells ($CD4^+$ / $CD8^+$ or $CD3^+$) and NK cells ($NK1.1^+$), which are derived from HSCs and HPCs in BM and spleen. Therefore, further estimate of the HSCs and HPCs compositions in BM and spleen is important to know the early alterations of hematopoiesis.

3.8 Enzyme-linked immunosorbent assay (ELISA)

ELISA is a quantitative immunoassay to test specific protein levels in liquid samples. It works as “sandwich” binding, where the 96-well plate is pre-coated with specific antibody for the test antigen, after exposure the specific antigen in the liquid samples to the plate for one

hour, the extra liquid is washed away. A specific enzyme-linked antibody is then added to bind to the test antigen in the 96-well plate. After another incubation followed by the removal of the extra enzyme-linked antibody, the stop solution is applied to each well and the color of absorbent could be measured under ELISA reader at 450 nm. The concentration of the testing antigen in the samples is further calculated based on standard curve. Due to the high sensitivity and specificity of ELISA assay, we used this technic to quantify the amount of THPO in mouse blood and BM as described in paper **I**.

3.9 Quantitative RT-PCR and single cell RT-PCR

PCR is a specific technique that can amplify the RNA transcripts of different tissues *in vitro* (Maheaswari et al., 2016). In attached paper **I** and **II**, to better understand the underlining mechanisms of BM niche in MPN and AML developments, we performed real-time quantitative PCR (Q-PCR) on freshly sorted BM niche cells, including MSCs, MPCs, $CD51^-$ $Sca1^-$ stromal cells, and endothelial cells. Due to the very few MSCs (less than 5000 cells in total) obtained from BM samples, we used RNeasy Micro Kit (Qiagen) for RNA isolation. We chose *Hprt*/*HPRT* as the housekeeping gene for QPCR analysis due to its stable and uniform expression in the BM stromal cells of the control and experiment mice. In paper **I** and **II**, we performed Q-PCR using TaqMan probes, which contained a fluorophore in the oligonucleotide. During amplification, fluorophore is released from the oligonucleotide and activated with a fluorescence, which can be detected by Q-PCR equipment. Given the specific hybridization between target gene and probe, TaqMan Q-PCR is considered as a low background and very sensitive method for detecting gene expressions. In addition, we performed Q-PCR to monitor the alterations of *Sca1* expression using SYBR Green I PCR kit in paper **III**. In paper **IV**, we applied single-cell RT-PCR as described (Qian et al., 2013) to detect BCR/ABL expression and genes involved in leukotriene signal pathways.

3.10 RNA sequencing and data analysis

RNA sequencing is a technique that provides a comprehensive gene expression profile of specific cell populations (Ingolia et al., 2012; Wang et al., 2009). Compared with microarray, RNA sequencing is more precise in transcriptome profiling including non-coding RNA in addition to coding RNA. RNA sequencing data can provide the information of new gene candidate to further identify their roles in pathogenesis of disease (Jabbari et al., 2012). In paper **I**, to further determine the molecular mechanisms involved in the pathogenesis of the MPN, we performed RNA sequencing to search for molecular alterations in different BM stromal cells from control and experiment mice. Due to the rare stromal population in BM, we extracted the RNA using RNeasy microkit. The cDNA library was prepared by using the Ovation® Ultralow Library Systems and subjected to 50 cycles of HiSeq 2000 or 76 cycles of NextSeq500 sequencing generating 20-30 million reads/sample.

All raw sequence reads available in FastQ format were mapped to the mouse genome (mm10) using Tophat2 combining with Bowtie2 (Kim et al., 2013; Langmead and Salzberg, 2012). PCR duplicates were removed using samtools (Li et al., 2009) after reads mapping.

Next, raw reads mapped to each gene were calculated using FeatureCounts from Subread package (Liao et al., 2014). Genes with Reads Per Kilobase of transcript per Million mapped reads (RPKM) values more than 0.1 were considered as being actively transcribed and proceeded to the analysis of Differential Gene Expression (DGE) (Mortazavi et al., 2008). DEseq2 was used to perform the analysis of DGE, where genes with raw read counts as input. The differentially expressed genes were identified by adjust *P* value for multiple testing using Benjamini-Hochberg correction with False Discovery Rate (FDR) values less than 0.2.

For Gene set enrichment analysis (GSEA), the read counts were first normalized by the Trimmed mean of M-values normalization method (TMM) (Robinson and Oshlack, 2010), and then the normalized read counts were performed on the GESA platform from Broad Institute (http://www.broadinstitute.org/gsea/index.jsp). The analyses were based on gene ontology (c5.all.v2.5.symbols.gmt), BioCarta (c2.biocarta.v2.5.symbols.gmt) and KEGG (c2.kegg.v2.5.symbols.gmt). Gene sets with a nominal *P* value < .05 and FDR < .25 were considered to be significantly enriched. All major computations were performed on resources provided by SNIC through Uppsala Multidisciplinary Center for Advanced Computational Science (UPPMAX) under Project b2014299.

3.11 Histology analysis

Histology study provides direct observations of pathogenic tissues. In attached paper **I** and **II**, to confirm the pathogenic alterations in the mice, we performed histology analysis on different tissues, such as peripheral blood, bone, spleen, and liver. Blood smear and cytospin slides were stained by May-Grünwald-Giemsa. Soft tissue were embedded in paraffin after fixation and dehydration. However, bones contained abundant of calcium and were decalcified with EDTA before being embedded in paraffin for section. Hematoxylin and eosin (HE) staining is the basic staining method for identification of pathogenesis alterations in tissue sections. Hematoxylin mainly stains the anion nuclei in the cells with blue color, whereas eosin is an acid dye and stained cytoplasm or connective tissue with pink red appearance. The 3D Histech Slide Scanner is used for scanning the HE stained bone sections. Pannoramic Viewer (3D HISTECH) is used for taking the images of blood, BM, and spleen sections. In addition, megakaryocytes are big in size and can be easily recognized from the tissue image. Therefore, we further calculate megakaryocytes numbers per area with the help from the software of Pannoramic Viewer.

3.12 Micro-Computed Tomography (μCT)

Micro CT can generate 3D structures of different tissues from live animals through X-ray beam, and provide direct morphologies of tissues without invasion. Given different organ densities, μCT form a distinct contrast between bones and surrounding soft tissues, which helps to understand the alterations of bone mass during pathogenesis. In addition, the microstructures of bones, such as bone volume, bone marrow volume, trabecular number, trabecular thickness can be further quantified by using the software Analyze 12.0. Therefore,

in paper **I**, we performed μCT to observe the alterations of mouse bones between control and experiment mice and quantified the alterations.

4 Results and Discussions

The thesis focuses on the roles of BM microenvironment on hematopoiesis and potential contributions of BM niche to myeloid malignancies. In study **I**, we determined that the Sipa1 loss-induced MPN was driven by the abnormal BM niche, providing an evidence of BM niche-induced myeloid malignancy using the *Sipa1*$^{-/-}$ mouse model. Next, in study **II**, we investigated the roles of BM niche in the AML development induced by MLL-AF9^{+} AML cells, and revealed that native BM MSPCs inhibited the AML progression but could be educated to form a leukemia niche favoring the AML growth. These two studies presented the important roles of BM stromal cells in the MPN and AML *in vivo*. Meanwhile, we also studied the effects of different culture conditions on the characteristics of BM MSCs *in vitro*. As indicated in study **III**, we showed that SCA1 expression on BM MSCs was reduced under non-adherent culture condition compared to adherent cultivation. In addition, in contract to the previous finding using liquid culture, the 5-LO inhibitors failed to exert significant inhibition of the growth of CML patient LSCs in long-term culture-initiating cell (LTC-IC) experiment, suggesting a possible protection of CML cells by stromal cells from the inhibitors as shown in study **IV**.

4.1 BM niche were altered in myeloid malignancies

In study **I** and **II**, by taking advantages of *Sipa1*$^{-/-}$ MPN mouse model, MLL-AF9^{+} AML mouse model, and AML NGS xenograft model, we investigated the alterations of BM niche in these myeloid malignancies.

To evaluate the involvement of BM niche in the progression of the MPN and AML, we phenotypically and functionally analyzed the BM niche cellular components in the mice when they developed to leukemia. In study **I**, the aged *Sipa1*$^{-/-}$ mice exhibited increased myeloid cells, enlarged spleens, BM megakaryocyte dysplasia, and leukocyte infiltration in their BM, indicating a mouse MPN phenotype of abnormal hematopoiesis. In these aged *Sipa1*$^{-/-}$ MPN mice, we detected increased BM MPCs but decreased MSCs, indicating altered BM cellular niche components in the MPN mice. *In vitro* multi-lineage differentiation assay implied increased adipogenic but declined osteogenic differentiation capicapies of the MSCs from aged *Sipa1*$^{-/-}$ MPN mice. These results indicated that BM stromal cells were phenotypically and functionally altered in the aged *Sipa1*$^{-/-}$ mice when they developed MPN. In study **II**, the AML mice displayed life threatening symptoms along with dramatic increased myeloid cells in blood and splenomegaly about 27-30 days after transplantation. We found that both frequencies and absolute numbers of BM MSCs, MPCs, and endothelial cells were increased in the AML mice. *In vitro* CFU-F assay of total BM mononuclear cells further confirmed the increased BM MSC numbers. To further understand the functional alterations of the AML BM MSCs, we performed *in vitro* multi-lineage differentiation assays, where we detected enhanced adipogenic and osteogenic differentiation capacities of AML BM MSCs. These results indicated that the AML BM stromal cells were phenotypically and functionally altered after the mice developed symptomatic AML. To dynamically monitor the alterations of the BM stromal cells, we analyzed the AML mice at different time point after

transplantation of AML cells. Interestingly, the phenotype of AML BM stromal cells seemed like normal at early stage after AML cell transplantation, but displayed alterations around 10 days before they showed AML symptoms. Furthermore, the dynamic alterations of AML BM stromal cells exhibited a liner correlation with the AML engraftment in the mice. Accordantly, we also detected similar phenotypic alterations of patient AML cells in NSG mice, suggesting that primary AML cells from patients induced similar alterations to their BM niche. Together, these results indicated phenotypic and functional alterations of the BM niche in the mice with myeloid malignancies.

4.2 The BM niche alterations in the *Sipa1*$^{-/-}$ mice prior to the MPN development

In study **I**, given the alterations of both BM hematopoietic cells and the stromal cells in the aged *Sipa1*$^{-/-}$ mice with MPN, we wondered whether the alterations of the BM stromal cells were attributed to the intrinsic *Sipa1* loss in the BM niche or the remodeling by malignant hematopoietic cells. To address this question, we characterized the BM niche of 3-month old young *Sipa1*$^{-/-}$ mice before the initiation of MPN. Interestingly, we detected increased BM MSCs and MPCs in the young *Sipa1*$^{-/-}$ mice although the hematopoietic cells were still normal. *In vitro* multi-lineage differentiation assay presented increased adipogenic but declined osteogenic differentiation capacities of the MSCs from the young *Sipa1*$^{-/-}$ mice, which were similar to that from the aged *Sipa1*$^{-/-}$ MPN mice. Furthermore, *in vitro* LSK HSPCs co-cultured with freshly sorted BM stromal cells from *Sipa1*$^{-/-}$ and *Sipa1*$^{+/+}$ mice displayed an enhanced capacity of the *Sipa1*$^{-/-}$ MSCs and MPCs in promoting myeloid cell differentiation. These data indicated that the BM niche of young *Sipa1*$^{-/-}$ mice altered before the onset of MPN, which was probably due to the intrinsic loss of *Sipa1* in BM niche, which might further led to MPN with time.

4.3 Dysfunctional BM niche resulted in the MDS/MPN

Previous study reported that SIPA1 expressed in mouse HSPCs (Ishida et al., 2003). Therefore, in study **I**, we designed transplantation experiments to determine whether *Sipa1* deficiency in the hematopoietic cells or BM niche led to the development of the MDS/MPN. We first transplanted CD45 beads enriched CD45.2^{+} cells from young *Sipa1*$^{-/-}$ mice, where the hematopoiesis remained normal, to 8-10 week old CD45.1^{+} *Sipa1*$^{+/+}$ recipient mice which received lethally irradiation (9.5 Gy). However, we did not detect any abnormal hematopoiesis in the recipient mice, indicating that the *Sipa1*$^{-/-}$ hematopoietic cells from young adult mice alone could not induce the MPN in a normal BM niche. We next transplanted CD45 beads enriched CD45.1^{+} cells from *Sipa1*$^{+/+}$ mice to CD45.2^{+} young *Sipa1*$^{-/-}$ mice and age-matched *Sipa1*$^{+/+}$ mice under sublethally irradiation (6 Gy). Expectedly, in the *Sipa1*$^{-/-}$ recipients, we detected increased donor-derived myeloid cells in the PB and BM, increased donor-derived GMPs and MEPs in BM, and increased donor-derived CMPs and GMPs in the spleen. Although sublethally irradiation could not completely clear up endogenous hematopoietic cells of the recipient mice, but the leftover endogenous-derived myeloid cells did not present any difference between *Sipa1*$^{+/+}$ and *Sipa1*$^{-/-}$ recipients. In addition, similar transplantations were performed on lethally irradiated *Sipa1*$^{+/+}$ and *Sipa1*$^{-/-}$

recipients. We observed even more severe phenotype of the MDS/MPN in the *Sipa1*$^{-/-}$ recipients, presenting reduced survival, increased myeloid cells in PB, BM hypercellularity and megakaryocyte hyperplasia, increased BM MEPs, splenomegaly, and increased HSPCs in spleen. The spleen cells from the primary *Sipa1*$^{-/-}$ recipients were then transplanted to the sublethally irradiated secondary *Sipa1*$^{+/+}$ recipients, which exhibited MDS-like phenotype at 6 month after transplantation. These results suggested that *Sipa1*$^{-/-}$ BM niche could induce the MDS/MPN in the mice.

4.4 Suppressive roles of native BM MSCs in AML development

BM Ebf2 expressing stromal cells have been identified as enriched MSC population with CFU-Fs with a frequency of 1 in 6 (Qian et al., 2013). In study **II**, to evaluate roles of Ebf2^{+} cells in the AML development, we first utilized an Ebf2-reporter mouse model (*Ebf2-Egfp* mice) as the recipient for AML transplantation. We next took the advantage of triple transgenic *Ebf2-Egfp* x *Ebf2-Cre*^{ER} x *Rosa 26-*^{loxp}*Stop*^{loxp}*-Tomato* mice to determine Ebf2^{+} cells-derived progeny during the AML development. Interestingly, we found that both Ebf2^{+} cells and Ebf2^{+} cells-derived progeny were increased in the MLL-AF9^{+} AML mice, and Ebf2^{+} cells could give rise to all fractions of BM stromal cells, indicating that Ebf2^{+} cells might be a key niche element contributing to the remodeling of BM niche during AML development. To functional test our hypothesis regarding the critical roles of Ebf2^{+} cells in AML development, we utilized transgenic *Ebf2-Cre*^{ER} x *Rosa26-*^{loxp}*Stop*^{loxp}*-Dta* mice as the recipients for establishing AML model. Surprisingly, we observed an overall shorter survival of the AML mice with the depletion of Ebf2^{+} cells. All together, our data suggested that Ebf2^{+} cells were critical BM cellular niche components and might act as a suppressor for the AML development.

4.5 Deregulation of HSC niche factors in the development of myeloid malignancies

Here we showed that myeloid malignancies could be induced from both abnormal BM niche (study **I**) and malignant hematopoietic cells (study **II**). We next investigated how the BM niche contributed to the leukemia.

In study **I**, to further understand the underlying molecular mechanisms of the *Sipa1*$^{-/-}$ BM niche-driven MDS/MPN, we performed RNA sequencing analysis on freshly sorted BM MSCs, MPCs, and endothelial cells of young adult *Sipa1*$^{+/+}$ and *Sipa1*$^{-/-}$ mice. Gene set enrichment analysis of the *Sipa1*$^{-/-}$ MSCs and endothelial cells exhibited up-regulated *Ras* and *Rap1* signaling due to *Sipa1* deficiency. Pro-inflammatory cytokines, such as TGF-β and IL6/JAK/STAT3 signaling, were increased in the *Sipa1*$^{-/-}$ MSCs, whereas TGF-β and TNFα signaling were enriched in the *Sipa1*$^{-/-}$ endothelial cells, indicating inflammation mediated MDS/MPN in the *Sipa1*$^{-/-}$ mice. Furthermore, *Cxcl12*, *Kitl*, and *Angptl1* genes, which were critical for maintaining normal hematopoiesis, were reduced in the *Sipa1*$^{-/-}$ MSCs and MPCs, indicating impaired hematopoietic supportive functions of the *Sipa1*$^{-/-}$ BM niche. *Il7*, a lymphokine, was detected a reduction in the *Sipa1*$^{-/-}$ MPCs, indicating impaired lymphogenesis in the *Sipa1*$^{-/-}$ mice. Reduced *Dicer1* expression, which could led to MDS in

mice (Raaijmakers et al., 2010), was also found in the *Sipa1*$^{-/-}$ MPCs. Reduced *Runx2* in the *Sipa1*$^{-/-}$ MSCs and MPCs implied an impaired osteogenic differentiation capacity, which further confirmed the declined osteoblast differentiation of the *Sipa1*$^{-/-}$ MSCs *in vitro*. In addition, *Thpo*, which was critical for HSC maintenance (Qian et al., 2007), was increased in the *Sipa1*$^{-/-}$ MPCs, and the increased THPO protein was further confirmed in BM plasma in the *Sipa1*$^{-/-}$ mice, indicating an altered THPO signaling in the *Sipa1*$^{-/-}$ MPCs.

In study **II**, to understand the underlying molecular mechanisms of BM niche to AML progression, we studied the gene expressions on freshly sorted BM stromal cells after the mice developed symptomatic AML. The BM MPCs have been shown as the main sources for the productions of cytokines and growth factors in the BM niche (Mendez-Ferrer et al., 2010). The BM MPCs of the AML mice were detected with impaired hematopoietic maintenance gene expressions, such as *Cxcl12*, *Kitl*, and *Angptl1*. Furthermore, the AML MPCs exhibited a decline of *Il7* and *Col1a1* expression, but an increase of *Spp1*/*Opn*, *Il6*, *Lama4*, and *Jag1* expression. In addition, the AML MSCs showed a reduction of *Angptl1* and *Col1a1* expression, but an increase of *Lama4*, *Lama5*, *Il6*, and *Fabp4* expression; while AML endothelial cells presented an increase of *Tgfb1* and *Lama5* expression. These results indicated that inflammatory cytokines (such as *Il6*, *Spp1* and *Tgfb1*), matrix proteins (such as *Lama4*, *Lama5*, and *Col1a1*), Notch signaling (*Jag1*), and hematopoietic maintenance growth factors (such as *Cxcl12*, *Kitl*, and *Angptl1*) from the BM niche were involved in the AML progression. The dynamic gene expression assay further revealed that most of the gene alterations were correlated with AML burden, and indicated that early interventions of these gene expressions might affect AML progression.

Abnormal BM niche-induced MDS/MPN and leukemic cells-induced AML indicated different origin of myeloid malignancies. However, from the gene expression profiles, we found that the hematopoietic maintenance growth factors including *Cxcl12*, *Kitl*, and *Angptl1* were declined whereas inflammatory cytokine signaling were increased in the altered BM niche cells of the two leukemic mouse models. These results indicated that targeting on BM niche cells or niche factors might provide new treatments for MDS/MPN and AML.

4.6 Phenotype alteration in BM MSCs in culture

MSCs have been shown to change their phenotypes under different microenvironment (Qian et al., 2012; Tormin et al., 2011). SCA1 has been identified as a mouse MSC marker expressed on both freshly isolated MSCs and cultured MSCs. In study **III**, we characterized the SCA1 expression of mouse BM MSCs under adherent and non-adherent cultures, and detected a reduction of the SCA1 expression on the MSCs under non-adherent culture for 72 hours compared to that in adherent cultivation. In addition, the MSCs from non-adherent culture regained the SCA1 expression after being reseeded into adherent plastic culture for 5 days, indicating non-adherent culture could temperately down-regulate the SCA1 expression on MSCs. This study provided another evidence of culture-related phenotypic alteration of MSCs.

4.7 Possible protection of LSCs by stromal cells from pharmaceutical therapy

ALOX5 inhibitors have been applied to a clinic trial based on the promising results from the CML mouse experiments, where the application of ALOX5 inhibitor impaired the mouse CML stem cells and prolonged the survival of the CML mice (Chen et al., 2009). In study **IV**, we examined the expressions of leukotriene signaling molecules of CML leukemic stem and progenitor cells and evaluated their survival using pharmaceutical inhibitors targeting ALOX5 or cysteinyl leukotriene receptor 1 *in vitro*. However, the ALOX5 inhibitors failed to exert significant inhibition of the growth of CML patient LSCs in LTC-IC experiment. In contract to the previous study that CML cells were inhibited in liquid culture, our study suggested a possible protection of CML cells by stromal cells from the inhibitors *in vitro*.

5 Concluding Remarks

This thesis has phenotypically, functionally and molecularly demonstrated the altered BM niche and their involvements in the development of myeloid malignancies. By using mouse models, paper **I** and **II** provided evidence for distinct roles of BM niche in initiation and progression of myeloid disease. In paper **I**, we demonstrated that the loss of *Sipa1* in mice led to BM niche alterations before the onset of MDS/MPN, and confirmed that the loss of *Sipa1* in the BM niche, but not in the hematopoietic cells resulted in the MDS/MPN. In paper **II**, we determined that native BM niche suppress the AML development, but could be gradually altered by leukemic cells favoring the AML growth. These two studies revealed the underlying mechanisms of the impaired BM niche-induced MDS/MPN and the AML-educated BM niche with self-reinforcing loops. Additionally, paper **III** illustrated that non-adherent culture could reduce the SCA1 expression of mouse BM MSCs. Finally, by single cell assays of CML LSCs, our study (paper **IV**) suggested that targeting leukotriene signaling ALOX5 and CYSLT1 with leukotriene antagonists was not helpful for suppressing CML growth in LTC-IC assay.

Collectively, we phenotypically, molecularly, and functionally characterized leukemic BM niche and determined the niche alterations and contributions to different myeloid malignancies. In addition, we revealed the underlying mechanisms of BM niche-induced MDS/MPN in mice, BM niche involvement in AML development *in vivo*, and potential protection of CML cells in culture. Targeting BM niche factors or restoring BM niche components might be new therapeutic strategies for these myeloid malignancies.

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